ORIANA ALEXANDRA DE PAIXÃO PRAÇAS MARQUES

IRON AND RELATED PROTEINS IN THE BREAST CANCER MICROENVIRONMENT: EXPRESSION PATTERNS IN EPITHELIAL AND STROMAL INFLAMMATORY CELLS AND ASSOCIATION WITH CLINICOPATHOLOGICAL MARKERS OF BEHAVIOR AND PROGRESSION

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But without it we go nowhere."

Carl Sagan, Cosmos (1980)

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ABBREVIATIONS

ADH – Atypical Ductal Hyperplasia
ALAS - AminoLevulinic Acid Synthase
APES - 3-AminoPropyltriEthoxySilane
Arg1 – Arginase 1
ASR – Age-Standardized Rate
Bcrp – Breast cancer resistance protein
BCS – Breast-Conserving Surgery
Bmp - Bone morphogenetic protein
BpT - 2-Benzoylpyridine Thiosemicarbazone
BRCA – BReast CAncer genes 1 and 2
CAF - Cancer-Associated Fibroblast
CCL - Chemokine (C-C motif) Ligand
CCL2 - Chemokine (C-C motif) Ligand 2
CCR2 - C-C Chemokine Receptor type 2
CD – Cluster of Differentiation
CDC – Cell Division Cycle
CHO – Chinese Hamster Ovary
ChT – ChemoTherapy
CI – Confidence Interval
CISH - Chromogenic *In Situ* Hybridization
CK – CytoKeratin
COX - CycloOXygenase
Cp - Ceruloplasmin
CSF-1 - Colony Stimulating Factor 1
CSF1R - Colony Stimulating Factor 1 Receptor
CXCL - C-X-C chemokine Ligand
DAB - DiAminoBenzidine
DCIS – Ductal Carcinoma *In Situ*
Dcyt B - Duodenal cytochrome B
D-exo - Desferri-exochelin
DFO – DesFerriOxamine
DMBA - 7,12-DiMethylBenz(a)Anthracene
DMT - divalent metal transporter
DpT - Di-2-pyridylketone Thiosemicarbazone
e.g. – *exampli gratia*
ECM – ExtraCellular Matrix
EGF - Epidermal Growth Factor
EGFR - Epidermal Growth Factor Receptor
EMT - Epithelial-to-Mesenchymal Transition
ER – Estrogen Receptor
ERE – Estrogen Responsive Element
ERK - Extracellular signal-Regulated Kinase
ET – Endocrine Therapy
FEA - Flat Epithelial Atypia
Fe-S – iron-sulfur
FFPE - Formalin-Fixed Paraffin-Embedded
FGF2 - Fibroblast Growth Factor 2
FGFR2 - Fibroblast Growth Factor Receptor 2

FISH - Fluorescence *In Situ* Hybridization
FLVCR - Feline Leukemia Virus subgroup C Receptor
FoxP3 - Forkhead box P3
FPN – FerroPortiN
FT - FerriTin
GADD45 – Growth Arrest and DNA Damage-inducible 45
Gal1 - Galectin-1
GOX - Glycolyate Oxidase
H – Heavy
H&E – Hematoxylin & Eosin
HAO - HydroxyAcid Oxidase
HCP1 - Heme Carrier Protein 1
HER2 - Human Epidermal growth factor Receptor 2
HGF - Hepatocyte Growth Factor
HH – Hereditary Hemochromatosis
HIF - Hypoxia Inducible Factor
HKa - cleaved High molecular weight Kininogen
HO - Heme-Oxygenase
HPF – High Power Field
HRT – Hormone Replacement Therapy
HUT – Hyperplasia of Usual Type
IBC – Invasive Breast Cancer
IDC – Invasive Ductal Carcinoma
IFN – InterFeroN
IGF - Insulin Growth Factor
IL – InterLeukin
IL-6R – InterLeukin 6 Receptor
iNOS - inducible Nitric Oxide Synthase
IRE – Iron Responsive Element
IRGS - Iron Regulatory Gene Signature
IRP - Iron-Regulated RNA binding Protein
KO - KnockOut
L - Light
LCIS - Lobular Carcinoma *In Situ*
LIP – Labile Iron Pool
LMW – Low Molecular Weight
LOH - Loss Of Heterozygosity
LPS - LipoPolySaccharide
mAb – monoclonal Antibody
MAPK - Mitogen-Activated Protein Kinase
MDSC - Myeloid Derived Suppressor Cell
MHC - Major Histocompatibility Complex
MIF - Macrophage migration Inhibitory Factor
MMP – Matrix MetaloProteinase
MNU - 1-Methyl-1-NitrosoUrea
MRI - Magnetic Resonance Imaging
mTOR - mammalian Target Of Rapamycin
NADPH - Nicotinamide Adenine Dinucleotide PHosphate-oxidase
NDRG1 - N-myc Downstream ReGulated 1
NF-κB - Nuclear Factor-κB
NHANES - National Health And Nutrition Examination Survey
Nramp - Natural resistance-associated macrophage protein
NSABP - National Surgical Adjuvant Breast and Bowel Program

NST - No Special Type
NTBI - Non-Transferrin Bound Iron
OXTR - OXyTocin Receptor
PARP - Poly (ADP-Ribose) Polymerase
PBS – Phosphate Buffered Saline
PCBP1 - Poly(rC)-Binding Protein 1
PCNA - Proliferating Cell Nuclear Antigen
PCR – Polymerase Chain Reaction
PDGF - Platelet-Derived Growth Factor
PDWA - Proliferative Disease Without Atypia
PHD - Prolyl Hydroxylase Domain
PI3K - PhosphatidylInositol 3'-Kinase
PIH - Pyridoxal Isonicotinoyl Hydrazone
PR – Progesterone Receptor
RFLP - Restriction Fragment Length Polymorphism
ROS – Reactive Oxygen Species
RR - Ribonucleotide Reductase
RT – RadioTherapy
sc - subcutaneous
SIH - Salicylaldehyde Isonicotinoyl Hydrazone
Slc40a1 - Solute Carrier Family 40 (Iron-Regulated Transporter), Member 1
SMA - Smooth Muscle Actin
Steap3 - Six-transmembrane epitelial antigen of the prostate 3
TAM – Tumor-Associated Macrophage
TDLU - Terminal Duct-Lobular Units
Tf – Transferrin
TFR - Transferrin Receptor
TGF-β - Transforming Growth Factor β
Th1 or Th2 – Type I (or II) Helper
TIBC - Total Iron Binding Capacity
TLR - Toll-Like Receptor
TMA – Tissue MicroArray
TN – Triple-Negative
TNF - Tumor Necrosis Factor
TNM - Tumor-Node-Metastasis
top2A - DNA topoisomerase IIA
Treg – regulatory T-cell
Triapine - 3-aminopyridine carbalhdehyde thiosemicarbazone
UTR – UnTranslated Region
VEGF - Vascular Endothelial Growth Factor
WAF1 - Wild-type Activating Fragment-1
WBC - Whole Blood Cell
WHO - World Health Organization
y. – years
β2m - β2-microglobulin

ABSTRACT

Iron is an essential functional element for several biologic processes, such as energy production and intermediate metabolism. However, when in excess, it can be toxic and produce free radicals. Deregulation of iron homeostasis has been consistently linked to the pathogenesis of chronic diseases. Cancer cells are thought to have higher requirements for iron. The importance of iron in tumor cell proliferation is demonstrated by the higher numbers of transferrin receptors than their 'normal' counterparts and by the fact that some iron chelators can suppress the growth of aggressive tumors.

It is generally accepted that invasive breast cancer develops from benign disease. Nevertheless, the progression from carcinoma *in situ* to invasive cancer remains poorly understood. While genetic and epigenetic alterations known to regulate cell proliferation, differentiation and/ or survival are the most likely initiators of breast carcinogenesis and since most ductal breast carcinoma cells are weakly invasive *in vitro* it is likely that stromal cell responses in premalignant stages may facilitate progression to invasive cancer. In this respect, the contribution of leukocytes to the regulation of the tumor microenvironment iron regulation had not been addressed before. Given the fact that circulating leukocytes besides taking up iron for their own survival are capable of exporting it in some conditions, we hypothesized that stromal inflammatory cells, by constituting a potential iron delivery system, may have an important role in breast cancer progression.

In the present study, we evaluated the cellular expression of iron-related proteins in the context of breast cancer microenvironment by immunohistochemistry and flow cytometry and analyzed possible association with tissue iron deposition and HFE genotype.

Results of the work showed that, as expected, breast cancer epithelial cells present an ‘iron-utilization phenotype’ with an increased expression of hepcidin and transferrin receptor 1 (TFR1), and decreased expression of ferritin (FT). In turn, lymphocytes and macrophages infiltrating primary tumors and from metastized lymph nodes display an ‘iron-donor’ phenotype with increased expression of ferroportin 1 (FPN1) and FT complemented by an activation status, as reflected by a higher expression of TFR1 and hepcidin. This phenotype is supported by the higher percentage of breast carcinomas presenting iron accumulation in stromal inflammatory cells. Furthermore, the deregulated expression of iron-related proteins in lymphocytes and macrophages is also associated with hormone receptor status and tumor size.

The chemokine (C-C motif) ligand 2 (CCL2) has an established role in leukocyte recruitment into the tumor microenvironment and has been recently described as a modulator of tissue iron levels. Epithelial CCL2 expression, which is increased in breast carcinomas samples, was also significantly higher in samples presenting iron deposition in stromal inflammatory cells. In fact, besides being associated with an increased infiltration of lymphocytes and CCL2-positive macrophages, its expression was also correlated with FPN1 expression in lymphocytes. These relationships suggest the existence of a paracrine signaling pathway where CCL2 may play an indirect role regulating tumor iron nutrition and progression. The median CCL2 expression in epithelial cells is not significantly different between patients presenting the HFE p.C282Y or p.H63D variants. However, p.C282Y/H63D compound heterozygous invasive carcinoma patients exhibit a higher expression of hepcidin in lymphocytes and macrophages and of TFR1 in all the cell types analyzed.

Surprisingly, the expression of iron-related proteins is not significantly different between benign and malignant mammary gland lesions obtained from cats and dogs. The lack of cancer cells' higher iron requirements may be explained by the fact that normal mammary gland from both species present strong iron deposition, unlike what happens in human samples.

In conclusion, our results reinforce the need to study the tumor microenvironment in breast cancer, while extending the knowledge of the contribution of immune cells to the local iron homeostasis in the tumor microenvironment context. Furthermore, the replication of the original study to other animal models allowed us to detect important physiological differences regarding the human breast tissue, demanding a more insightful knowledge of systemic and local iron homeostasis in cats and dogs.

RESUMO

O ferro é um elemento essencial em vários processos biológicos, incluindo a produção de energia e o metabolismo secundário. No entanto, quando em excesso, pode ser tóxico e levar à produção de radicais livres. A desregulação dos mecanismos homeostáticos dos níveis de ferro tem sido associada à patogénese de doenças crónicas, como o cancro. A importância do ferro na proliferação celular neoplásica é confirmada pela evidência de número superior de receptores da transferrina 1 em comparação com células não neoplásicas, e pelo facto de alguns agentes quelantes de ferro serem capazes de suprimir o crescimento de tumores mais agressivos.

É geralmente aceite que o carcinoma invasor da mama se desenvolve a partir de alterações benignas na mama. No entanto, a progressão de carcinoma *in situ* para carcinoma invasor permanece relativamente incompreendida. Enquanto as alterações genéticas e epigenéticas que regulam a proliferação celular, diferenciação e/ ou sobrevivência desempenham um papel crucial na iniciação do processo de carcinogénese, durante a invasão as interacções com as células do estroma são essenciais, dado que as células de carcinoma da mama têm um potencial de invasão limitado *in vitro*. Neste contexto, a contribuição dos leucócitos para a regulação do ferro no microambiente tumoral ainda não foi abordada. Dado que os leucócitos são células capazes não só de internalizar ferro para a sua própria sobrevivência, mas também de o exportar em determinadas condições, levantámos a hipótese que as células inflamatórias do estroma poderiam contribuir para a progressão do carcinoma da mama através da distribuição local de ferro.

Neste estudo, avaliámos a expressão de proteínas envolvidas na regulação celular do ferro no microambiente tumoral da mama por imunohistoquímica e citometria de fluxo e analisámos possíveis associações com a deposição de ferro no tecido e com a presença de variantes do gene HFE.

Como descrito anteriormente, verificámos que as células epiteliais de carcinoma da mama apresentam um perfil de utilização de ferro, através uma maior expressão de hepcidina e receptor da transferrina 1, e uma menor expressão de ferritina. Por sua vez, linfócitos e macrófagos presentes no infiltrado inflamatório e em nódulos linfáticos metastizados exibem um perfil doador de ferro, com uma expressão aumentada de ferroportina 1 e ferritina. Simultaneamente, a expressão elevada de hepcidina e receptor de transferrina 1 sugerem a activação destes tipos celulares. Este fenótipo é corroborado

pelo facto que, comparativamente com amostras de mamoplastias estéticas de redução, uma maior percentagem de casos de carcinoma da mama apresenta acumulação de ferro em células inflamatórias do estroma. Adicionalmente, alterações na expressão destas proteínas reguladoras do metabolismo celular do ferro estão associadas com o *status* dos receptores hormonais e tamanho do tumor na altura do diagnóstico.

O CCL2 é uma citocina pertencente à família das quimiocinas C-C com um papel comprovado como recrutadora de leucócitos para o microambiente tumoral. Recentemente foi descrito que esta também seria capaz de modelar os níveis locais de ferro no tecido. A expressão de CCL2 nas células epiteliais, previamente descrita como aumentada em casos de carcinoma da mama e confirmado no nosso estudo, foi demonstrada aqui ser também significativamente superior nos casos que apresentam deposição de ferro nas células inflamatórias do estroma. De facto, para além da expectável associação com a infiltração de linfócitos e macrófagos CCL2-positivos, a expressão de CCL2 está também correlacionada com a expressão de ferroportina 1 nos linfócitos. Estas relações sugerem a existência de uma via de sinalização parácrina onde o CCL2 pode ter um papel indirecto na regulação da nutrição e progressão tumoral. Os níveis de expressão de CCL2 no tecido mamário não são significativamente diferentes em doentes com as variantes p.C282Y e/ou p.H63D do gene HFE. No entanto, doentes heterozigóticas compostas p.C282Y/p.H63D apresentam uma maior expressão de hepcidina em linfócitos e macrófagos do infiltrado inflamatório, bem como de receptor da transferrina 1 nos tipos celulares analisados.

Surpreendentemente, a expressão das proteínas reguladoras do metabolismo do ferro não é significativamente diferente entre lesões mamárias benignas e malignas em amostras de gatas e cadelas. A observação de que as células tumorais mamárias de gatas e cadelas não apresentam maiores exigências de ferro do que as suas congéneres benignas pode ser explicada pelo facto de que a glândula mamária normal destas espécies apresenta já uma forte acumulação de ferro, ao contrário do que se verifica na espécie humana.

Em conclusão, os nossos resultados reforçam a necessidade de estudar o microambiente tumoral no carcinoma da mama, enquanto ampliam o conhecimento sobre a contribuição das células do sistema imune para a regulação local do ferro, em contexto tumoral. Adicionalmente, a translação do estudo original em animais modelo de carcinoma da mama permitiu-nos detectar diferenças fisiológicas nas mamas das gatas e cadelas em relação à mama da mulher, levantando a necessidade de um conhecimento mais aprofundado sobre a regulação sistémica e local da homeostasia do ferro nestas espécies.

Chapter 1

General Introduction

1.1. Breast Cancer

1.1.1. The Human Breast

The breast is a dynamic organ that suffers several changes during the embryonic development, puberty, pregnancy, lactation and involution. The development of the ductal system is termed branching morphogenesis, and although it starts in the fetus, it is only completed during puberty when hormonal stimulation triggers differentiation [1, 2]. Under hormonal influence, complex reciprocal interactions between epithelial and stromal cells drive the alterations observed in the mammary gland in a woman's lifetime.

The adult female breast lies on the anterior chest wall, over the pectoralis major muscle, and is placed normally between the 2nd and 6th ribs of the chest wall in the vertical axis and between the sterna edge and the midaxillary line in the horizontal axis [3, 4]. The breast also extends superolaterally to the axilla [5].

The breast is composed by 15-20 lobes, each one drained by a collecting duct. These collecting ducts link the nipple with lactiferous sinus, that by its turn are connected to terminal duct-lobular units (TDLUs), the breast functional unit, by lactiferous and major ducts. Lobules consist of TDLUs, acini and their underlying, hormone-responsive, supporting stroma, consisting of various proportions of fibrous and adipose tissue [6] (Figure 1).

Except for a part of the collecting ducts near the nipple, composed of squamous epithelium, the ductal system consists of two main epithelial layers: an inner ductal layer composed of columnar luminal epithelial cells and an outer spindle-shaped myoepithelial cell layer. The epithelial cells of the inner layer are typically immunoreactive for low molecular weight cytokeratins (CK) 8, 18 and 19 and characterized by a cytoplasm with abundant organelles involved in secretion. The outer layer is characterized by the expression of high molecular weight CKs 5/6, anti-actin antibodies and p63, in close contact with the basal lamina [3, 4]. Myoepithelial cells are responsible not only by the maintenance of the ductal structure but also by assisting milk ejection [7]. The basal lamina in conjunction with the epithelial-myoepithelial cell layers and the surrounding zone of delimitating fibroblasts is denominated of epithelial-stromal junction [3]. Lymphocytes, plasma cells and macrophages normally lie in the interstices of this fibroblast network, which facilitates cell-cell interactions [8]. The intralobular stroma is more cellular and contains more vessels than the interlobular stroma, while the latter is more collagenized [3, 4]. The complex lymphatic network present in the mammary gland is drained, mostly,

to the axillary lymph nodes, which facilitates epithelial cell spread in the case of metastatic disease [9].

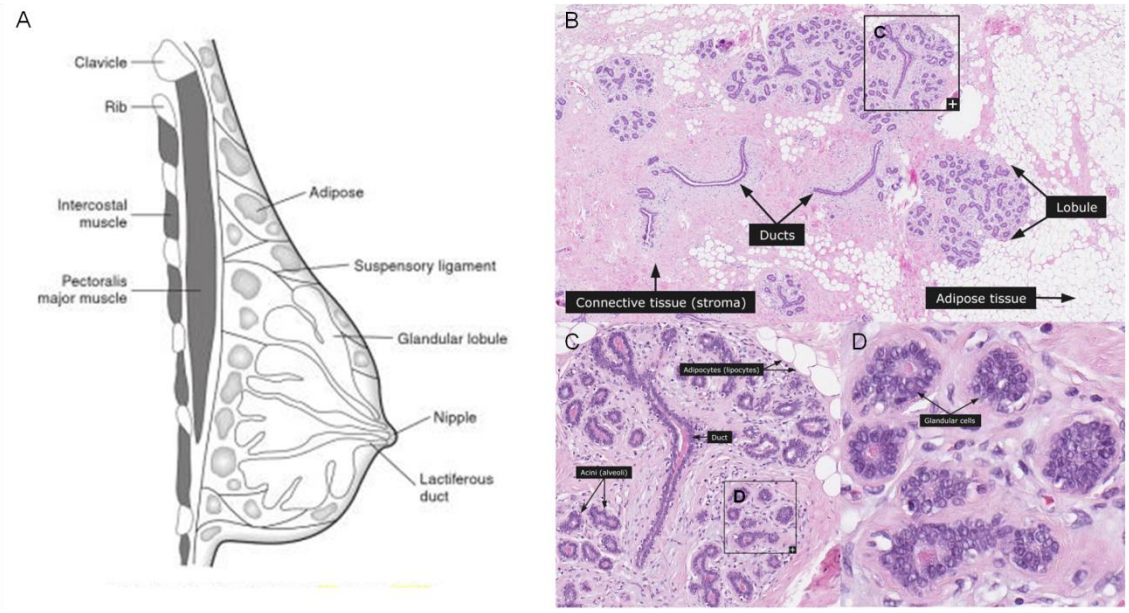

Figure 1. A. Schematic representation of the main anatomic features in an adult woman's breast. ***Adapted from [10]*****. B-D. Mammary gland characteristic histological elements, highlighting the ducts, connective and adipose tissue, lobules and acini.** ***Adapted from [2, 11].***

1.1.2. Breast Cancer

Breast female lesions are frequent. As fear of cancer is very common, use of image technology in screening is generalized and identifies most of the lesions, when no clinical manifestation still exists. If present, they manifest as palpable nodules or masses [12]. Although most of known epithelial benign and malignant lesions of the breast arise at the level of the TDLU other common breast diseases also have origin in other sites [13](Table 1).

Table 1. Origin sites for common breast diseases. ***Adapted from [13]*****.**

Nipple	▪ Paget's Disease ▪ Nipple Adenoma
Lactiferous Ducts	▪ Subsclerosing Duct Hyperplasia ▪ Duct Ectasia
Segmental and Subsegmental Ducts	▪ Solitary Intraductal Papilloma ▪ Duct Ectasia
Terminal Duct Lobular Units	▪ Cyst ▪ Epithelial Hyperplasia ▪ Noninvasive and Invasive Carcinoma

Fibrocystic changes are the most common alterations in the adult premenopausal women and thought to be caused by breast alterations during menstruation cycles [12]. Although of little clinical significance, some benign changes may confer an increased risk for the development of breast cancer [14-16].

Even though the exact mechanisms involved in the development of breast cancer remain unknown, the most commonly accepted model hypothesizes that invasive cancer starts in the TDLU [17] and progresses through pre-malignant breast disease by increasing cellular anomalies leading to exaggerated proliferation and atypia [18-20]. A proliferative growth advantage in flat epithelial atypia (FEA) can then give rise to atypical ductal hyperplasia (ADH) that may progress to ductal carcinoma *in situ* (DCIS) upon additional molecular alterations [21, 22]. Several epidemiologic studies support this stepwise model of progression in which invasiveness arises through the accumulation of abnormalities in benign breast diseases [14, 16, 23, 24]. Each stage is considered to result from genetic alterations in a transformed clonal lineage, eventually capable of invasion and metastasis [25]. Furthermore, matched genetic and epigenetic alterations are frequently found in proliferative diseases without atypia (PDWA), ADH, DCIS and invasive breast cancer (IBC) in the same breast [18, 26-28], supporting a sequential relation between precursor lesions and the IBC with which they are associated [25]. Surprisingly, morphologically normal terminal duct lobules adjacent to breast cancer may already present loss of heterozygosity (LOH) in genes critical for early progression of tumorigenesis [29]. However, the exact place and how DCIS progresses to IBC remains a matter of discussion, with two theories attempting to explain it (Figure 2). The theory of linear progression states that low-grade DCIS evolves linearly to high-grade DCIS and this

is the entity accountable for the progression to IBC [15, 30-32]. On the other hand, the theory of parallel disease hypothesizes that low-grade DCIS progresses to low-grade IBC, while high-grade DCIS evolves to high-grade IBC, implying a commitment of a particular type of DCIS to its IBC counterpart [33]. This model is supported by cytogenetic studies showing that specific patterns of chromosomal alterations in particular grades of DCIS correspond to distinct genomic changes in matching IBCs [34, 35] and that the degree of DCIS differentiation was correlated with that of the corresponding IBC [36]. However, the concept that these two models are mutually exclusive may underestimate the complexity of the process [37].

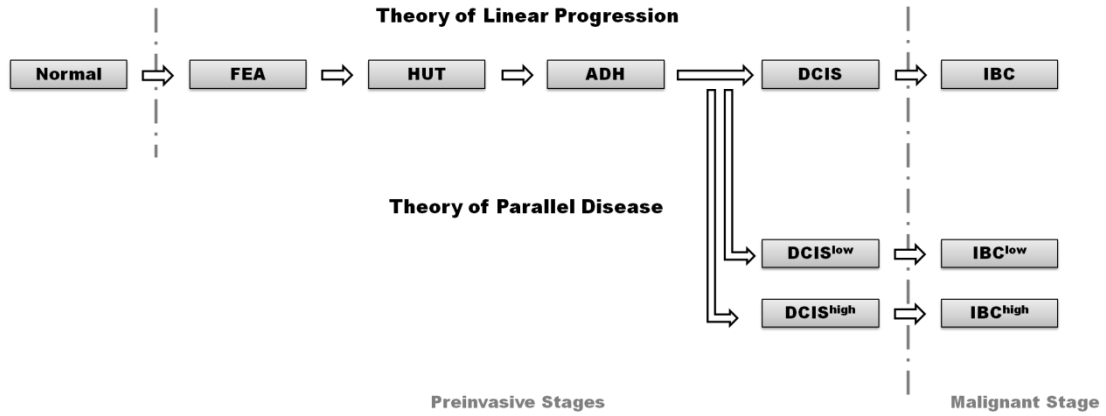

Figure 2. Stepwise model of breast cancer development highlighting the two views of progression from ductal carcinoma in situ to invasive breast carcinoma (Theory of Linear Progression and Theory of Parallel Disease). Abbreviations: FEA, flat epithelial atypia; HUT, hyperplasia of usual type; ADH, atypical ductal hyperplasia; DCIS, ductal carcinoma in situ; DCISlow, low-grade ductal carcinoma in situ; DCIShigh, high-grade ductal carcinoma in situ; IBC, invasive breast cancer; IBClow, low-grade invasive breast cancer; IBChigh, high-grade invasive breast cancer.

1.1.2.1. Epidemiology

Besides major efforts for disease prevention and treatment, cancer is still a main public health problem. In 2012, 3.4 million new cancer cases were diagnosed and 1.75 million people died of neoplastic disease worldwide [38]. Breast cancer is the most commonly diagnosed type of cancer, closely followed by colorectal cancer [38, 39].

In women, breast cancer is by far the most frequently diagnosed neoplasm, representing nearly 30% of the total, and ranks as the fifth cause of cancer-related death [38, 39]. Populations at higher risk of incidence and mortality are situated in Northern America, Western and Northern Europe (Figure 3A). Although presenting a slightly higher prevalence than the mean established for European Union countries, Portugal follows the

tendency, with breast cancer representing the most frequently diagnosed type of cancer and cause of cancer death in women (Figure 3B). In Portugal, in 2012, 6066 new cases of breast cancer were diagnosed, with 1570 women succumbing to the disease [39].

Although breast cancer mortality rates have been decreasing since the 1990's, specifically due to increasingly efficient screening regimens detecting the disease as early as possible, its high incidence demands a greater awareness and investment in treatment and education.

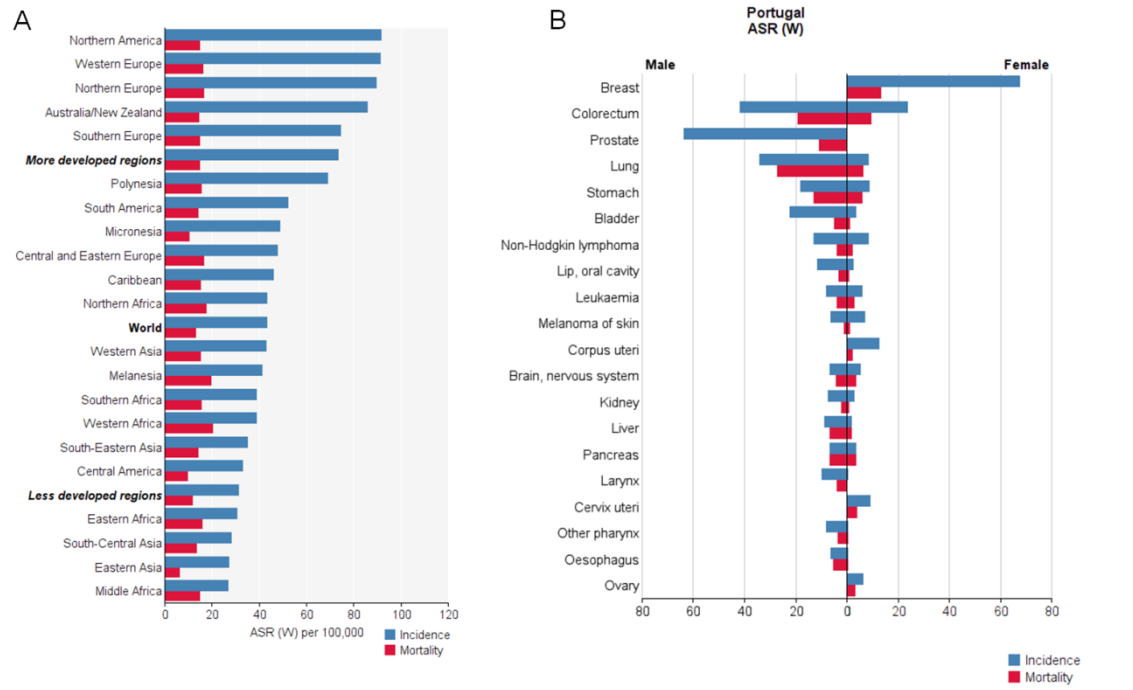

Figure 3. A. Breast cancer age-standardized rate (ASR) of incidence and mortality, per 100000 habitants, in the World. B. Age-standardized rate of incidence and mortality of the top 20 most common types of cancer in Portugal. ***From [39].***

1.1.2.2. Risk Factors

Several factors have been consistently associated with an increased risk for the development of breast cancer. Although the following do not deplete the list of possible influencing factors, they clearly highlight the multifactorial etiology of the disease. Breast cancer risk factors can be divided in two main groups. The first includes inherent factors such as age, gender and family history. The second relates to extrinsic factors influenced by the woman's lifestyle that may condition the neoplastic context to a certain degree [40]. Next, a table summarizing the main established risk factors for breast cancer is presented.

Table 2. Breast Cancer Risk Factors.

Intrinsic Factors	
Gender	▪ Breast cancer is predominantly diagnosed in women [41].
Personal History	▪ Previous history of DCIS, hormone receptor negative IDC and young age [42].
Genetic Predisposition	▪ Genes in which mutations increase risk: Breast cancer genes (BRCA) 1 and 2, P53, PTEN, STK11, CDH-1 [43-47].
Age	▪ Risk increases with age [48, 49].
Family History	▪ Increased in case of history of affected first-degree relatives, particularly at young age (<50 y.) [50, 51].
Other Breast Diseases	▪ Proliferative diseases, especially with atypia confer a substantial increased risk [15, 52].
Early Menarche	▪ 2 y. delay in menarche corresponds to a risk reduction of 10% [53].
Parity, age of full term pregnancy and breast-feeding	▪ Nulliparousity, parity at an older age (>35 y.) and absence of breast-feeding increases risk [49, 54, 55].
Age at menopause	▪ Every year delay increases the risk by 3% [53, 56].
Race	▪ Higher rate of mortality in Black women, due to higher incidence of triple-negative (TN) tumors [49].
Testosterone	▪ High circulating levels of testosterone in postmenopausal women increase risk [57].
Extrinsic Factors	
Hormone Replacement Therapy (HRT)	▪ Breast cancer risk is higher in hormone replacement therapy (HRT) users, especially when started close to menopause [58, 59].
Obesity	▪ Elevated risk with increasing weight, body mass index and hip circumference [58, 60].
Physical Activity	▪ Physical activity reduces risk in a dose-dependent manner [61, 62].
Alcohol Consumption	▪ Increased risk with binge drinking [63].
Radiation	▪ Radiation exposure at young age (<35 y.) increases risk [64-66].

1.1.2.3. Carcinoma *In Situ*

Carcinoma *in situ* is a non-obligate precursor of invasive carcinoma, characterized by an abnormal proliferation of epithelial cells confined in the ductal system by the basement membrane, without invasion of the surrounding stroma [67, 68]. The presence of malignant epithelial cells beyond the basement membrane is routinely recognized as individual cells or irregular nests through the stroma. The disruption of the myoepithelial layer may be demonstrated by the absence of myoepithelial cell markers such as p63, calponin or smooth muscle myosin [69].

Initially, carcinomas *in situ* were divided into lobular carcinomas *in situ* (LCIS) and ductal carcinomas *in situ* based on the assumption that these preinvasive lesions were generated either on lobules or on ducts, respectively. However, studies by Wellings and collaborators revealed that most of the lesions arised in the TDLU, marking the end of the distinction between ductal and lobular subtypes [17, 70, 71], which is merely morphological. The characteristic histological aspect of 'historic' LCIS is hallmarked by the absence of E-cadherin expression. However, it is not an exclusive aspect, with DCIS also demonstrating E-cadherin loss [72].

Through the observation of nuclear atypia DCIS can be further classified in low, intermediate and high-grade groups [21, 23, 68]. Historically, the architectural pattern of the DCIS was described and it correlated well with several tumor markers. Categories included non-comedo (cribiform, papillary, micropapillary and papillary) and comedo-subtypes [73]. The comedo subtype was characterized by the presence of central ductal necrosis and high-grade cells, and associated with estrogen receptor (ER) negativity, Human Epidermal growth factor Receptor 2 (HER2) amplification, p53 mutations and a high proliferation rate [74-79]. With the increasing number of diagnosed DCIS, became apparent that the classification in comedo/ non-comedo was not adequate to classify, for example lesions with central necrosis and low-grade cell pleomorphism. The need for refinement led to several proposed classification schemes until the one in effect today [80, 81]. Size of the lesion is also an important histological parameter to include in the breast pathology report given its clinical significance [68]. Until the 1980's DCIS was rarely diagnosed and represented less than 1% of detected breast cancers [82]. However, with the introduction of efficient mammography screening regimens detection of early breast cancer improved significantly. Nowadays, DCIS constitutes 20-25% of newly diagnosed breast cancer in the United States of America [83]. Implementation of screening mammography led to a significant reduction of the average size of DCIS lesion at presentation, diminishing from 60 to 10mm [84]. In spite of a clear increase in the

incidence of DCIS in the last 25 years, with concomitant improved treatments and outcomes, a consequent reduction in the incidence of invasive breast cancer has not occurred [83].

Most women do not present symptoms at the time of DCIS diagnosis, with the lesion being detected as a mammographic abnormality. Usual clinical symptoms include breast lumps, nipple discharge or Paget's disease [85]. Accurate diagnosis of DCIS and exclusion of invasion breast cancer is critical in order to determine the most adequate treatment [69].

Historically, DCIS treatment was achieved by simple mastectomy, with success rates over 99% [86]. However, mastectomy is associated with a poor body image and quality of life [87, 88]. Although it still remains a treatment option, especially in extensive DCIS [69], successful treatment of IBC with breast-conserving therapy suggests that DCIS may be overtreated [89-92].Thus, after promising results from the National Surgical Adjuvant Breast and Bowel Program (NSABP) B17 study, breast-conserving therapy was established as the standard of care for DCIS [90, 91]. The efficacy of radiation to reduce the risk of breast cancer recurrence after breast-conserving therapy has been clearly demonstrated [93-95], with a 15.2% reduced 10-year risk of any ipsilateral breast event [95]. Although randomized controlled trials describe a decrease in the number of ipsilateral and contralateral breast cancer events, the use of tamoxifen after breast-conserving surgery for DCIS remains controversial due to toxicity concerns. Since these studies have not demonstrated an increase in mortality, the National Comprehensive Cancer Network Guidelines recommended the use of tamoxifen for 5 years for ER-positive DCIS [96, 97].

1.1.2.4. Invasive Carcinoma

Invasive breast cancer constitutes a heterogeneous and complex disease that includes several pathologies, with different histological characteristics, as supported by the variable outcomes in diagnosed patients [23, 98]. The current definition of invasive breast cancer lies on the observation of malignant cancer cell spread, through breach of the myoepithelial cell layer and basement membrane to the adjacent stroma [99, 100].

Invasive carcinomas of the breast are classified according to specific morphological features. The majority of these are included in the category of 'invasive carcinoma, no special type' (NST) also known as invasive ductal carcinoma (IDC) [98, 101-103]. This group is very heterogeneous, in such a way, that the identification alone

does not provide prognostic and predictive information. Special type invasive carcinomas constitute approximately 25% of all breast cancers and display a particular morphology that is present in, at least, 90% of the tumor [101-103] (Box 1).

Box 1. Most common breast cancer histological subtypes, based on the World Health Organization classification of breast tumors. Adapted *from [101, 102].*
Invasive carcinoma, no special type
Invasive tubular and cribiform carcinoma
Medullary carcinoma
Invasive lobular carcinoma
Invasive mucinous carcinoma
Neuroendocrine carcinoma
Invasive papillary and micropapillary carcinoma
Metaplastic carcinoma
Apocrine carcinoma
Adenoid cystic carcinoma

Diagnosis is aimed preferentially at detection before invasion, once breast cancer has no lethal potential before that [104]. In concordance, eighteen European countries set regional or national-based screening mammography programs to detect breast cancer in a pre-invasive stage [105]. The initial line of diagnosis is based on physical examination, which should be done with the patient sitting upright. Inspection of breast anomalies (e.g. asymmetry and masses), skin changes and cervical, supraclavicular and axillary lymph node basins should be carried out carefully. Following diagnosis, staging and treatment decisions are based on diagnostic imaging and core or fine needle biopsies [106]. Mammography remains as the main pillar of breast cancer detection, either through screening, diagnostic mammograms after discovery of a palpable mass of or other symptom of breast disease or follow-up mammograms. As for DCIS, invasive carcinomas present as masses, asymmetric entities or calcification punctuations [104, 107, 108]. More recently, magnetic resonance imaging (MRI) has also been considered an important tool in breast cancer diagnosis and treatment, particularly in cases where mammographic evaluation is constrained by aesthetic augmentation, interpretation of inconclusive findings in mammography, ultrasonography or assessment of disease extent at the time of diagnosis, cases of familial breast cancer associated with BRCA mutations and lobular

cancers [106, 107]. Additionally, ultrasound may also be performed as a screening method in women at high risk for breast cancer that are not candidates for or do not have access to MRI and in cases of suspicion of axillary lymph node involvement. Furthermore, the fact that ultrasound allows for real-time imaging makes it an optimal tool during interventional procedures [107]. Besides imaging, disease pre-treatment must include a complete personal medical history and history of family breast/ovarian cancer, whole blood cell (WBC) count, liver and renal function assessment, alkaline phosphatase and calcium levels and menopausal status [106].

Final diagnosis is made based on the WHO classification [103] and the Tumor-Node-Metastasis (TNM) combinatory staging system, in order to classify the disease in one of five stages (0, I, II, III, IV). The latter includes information on the size of the primary tumor, status of regional lymph nodes and the presence or absence of distant metastasis at the time of diagnosis, and is essential to clinical decision-making to personalized patient therapy. Tumor size is defined based in radiological examination with histological confirmation [102]. In node-negatives patients, tumor size is considered the most powerful prognostic variable and critical regarding adjuvant treatment decisions [109]. Lymph node status is classified according to the extension of lymph node involvement. It is considered the most influential prognosis indicator and a critical determinant of adjuvant chemotherapy. Lymph node metastasis is associated with tumor size and decreased disease-free and overall survival [110, 111]. Presence or absence of distant metastasis at the time of diagnosis may be detected by patient symptomatology, clinical examination and radiological results [102]. Despite intensive screening programs, 6-10% of patients present metastatic disease when diagnosed [112, 113], and 30% of patients with early breast cancer will eventually develop distant metastasis [114]. Patients with distant metastasis present stage IV disease independently of tumor size and nodal status and a 5-year survival rate of approximately 22% [115]. Bone, lung, brain and liver are the most common breast cancer-metastized organs [116]. However, the histological type of tumor and TNM staging are not the only prognostic factors in breast cancer management. The classification of invasive breast cancer based on established biomarkers with prognostic significance, such as ER, Progesterone Receptor (PR) and HER2 constitutes standard practice. They provide additional prognostic and predictive value while aiding in adjuvant and chemotherapy regimen decisions [98, 102]. Accuracy and consistency in the evaluation of these biomarkers is of mounting importance, in order to guarantee comparable results that may follow therapy decision guidelines [106, 117, 118].

The most well-established biomarkers of prognostic, predictive and therapeutic importance in breast cancer are presented next.

- **Estrogen Receptor** – is a nuclear transcription factor that promotes normal and malignant epithelial cell proliferation when stimulated by estrogen through Cyclin D1 activation [119-121]. ER assessment is routinely done by immunohistochemistry (IHC) and evaluated in epithelial cell nuclei [122]. Although there is much debate surrounding the decision of the most appropriate threshold for classifying a tumor as ER-positive [123, 124], it is generally accepted that more than 1% of ER-positive cells is clinically relevant, once tumors even with a low expression of ER benefit from hormonal therapy when compared with ER-negative tumors [118]. Additionally, patients diagnosed with ER-positive tumors present higher free and overall survival [125, 126]. Tamoxifen was the first anti-estrogenic adjuvant therapy aimed at treating ER-positive tumors [98]. Its effects are due to blocking of estrogen stimulation and prevention of essential conformation changes needed for the association of coactivators, through ligation to the ligand-binding domain of ER [127, 128].

- **Progesterone Receptor** – PR, as ER, is also a nuclear transcription factor that stimulates the breast epithelium through progesterone [119, 120, 129]. Given that ER regulates PR expression [130, 131] it is thought that PR positivity implies ER positivity. However, a small percentage of invasive breast tumors do not have concordant ER and PR status [132, 133]. In this respect, it is important to identify these tumors as they might also benefit from hormonal therapy [134].

- **HER-2** – HER-2 is a transmembrane tyrosine kinase receptor of the ErbB family, whose coding gene (ERBB2) is located on chromosome 17q21 [135-137]. HER-2 signaling pathway activation leads to increased cell proliferation through the RAS-mitogen-activated protein kinase (MAPK) pathway and decreased apoptosis through the phosphatidylinositol 3'-kinase (PI3K)- AKT- mammalian target of rapamycin (mTOR) pathway [138]. HER-2 gene amplification, which occurs in 15-30% of invasive breast cancers [98, 102, 139], is strongly correlated with increased HER-2 protein expression. HER-2 amplification is a biomarker of poor prognosis once HER-2 positive tumors present a more aggressive phenotype, resistance to anti-hormonal and cytotoxic therapies and lower overall survival [140-142]. Although HER-2 positivity is related to an adverse prognosis, these tumors respond to therapies targeting the HER-2 proteins, such as trastuzumab and lapatinib [143, 144]. Although its antitumor effects are not fully understood, trastuzumab is thought to act by inhibiting receptor-receptor interaction, HER-2 cleavage and consequent production of an active truncated HER-2 fragment, increasing

receptor destruction by endocytosis and cytotoxicity, and immune activation [145-148]. HER-2 status is routinely assessed through immunohistochemistry, and fall into one of four categories (0, 1+, 2+, 3+) [117]. Tumors presenting no staining (0) or incomplete membrane staining in less than 10% of breast epithelial cells (1+) are considered HER-2 negative. Invasive breast tumors are considered HER-2 positive (3+) when at least 30% of tumor cells display complete membrane staining. Invasive breast cancers with equivocal immunohistochemistry results (+2) require testing by *in situ* hybridization (Silver *in situ* hybridization [SISH]) to confirm gene amplification [117, 149].

- **Ki-67** – the ki-67 antigen is a non-histone nuclear protein present during all active phases of the cell cycle, except G0 and early G1[150]. Given so, it is considered a marker of cell proliferation [151, 152]. The Ki-67 score is considered a valuable tool and a preferential method for the assessment of the proliferation index instead of counting mitoses and represents an independent prognostic parameter of disease-free and overall survival [153-155] while being able to predict response to preoperative chemotherapy [156]. Although the International Ki-67 in Breast Cancer Working Group has attempted to define guidelines for Ki-67 scoring and thresholds [157] its use in clinical practice remains limited due to interlaboratory variability [158].

- **p53** – The p53 gene is located on chromosome 17p13.1 [159] and encodes a 375 amino acid nuclear phosphoprotein involved in several critical pathways that regulates cell cycle arrest, apoptosis, DNA repair and senescence [160-163]. p53 mutations are the most common mutations in breast carcinomas (approximately 30%) [164]. However, its frequency and type of mutation varies greatly between tumor subtypes [165, 166]. p53 gene mutations result in an altered protein configuration and prolonged half-life which ultimately leads to accumulation of the mutated protein [167-169]. Expression of p53 mutant proteins is associated with higher tumor proliferation, early disease recurrence, chemotherapy resistance and lower overall survival in node-negative breast cancer patients [170-172]. Although nuclear mutant p53 protein accumulation may be detected by immunohistochemistry [173], as a surrogate for p53 gene mutations, no therapy takes this biomarker status in account [98].

1.1.2.4.1. Molecular Subtypes

As reflected by the variable outcome in patients, breast carcinoma heterogeneity is well acknowledged. The advent of the genomic era and development of even more sensitive technologies for the evaluation of gene alterations and protein expression led to

a better understanding of the biological basis underlying the observed differences [102, 174]. The study by Perou and collegues [175] presented as a turning point in breast cancer research by demonstrating that the molecular background of these tumors reflected its variability. Using microarray technology, they have studied 496 genes (nominated “intrinsic gene subset”) in 65 tumors from 42 individuals, and found several differences in specific signaling pathways and cellular components [101]. Through unsupervised hierarchical cluster analysis they were able to categorize breast tumors into biological subgroups that relate to the cell of origin of the tumor: ER-positive tumors (Luminal subtype), associated with the expression of typical breast epithelial genes and ER-negative tumors (HER-2 and basal/ triple-negative subtypes), expressing characteristic genes of basal/myoepithelial cells [175, 176]. A separate group, characterized by genes normally expressed in adipose tissue and basal cells, was designated as the normal breast subtype, and is thought to represent contamination from normal breast tissue [101]. Sorlie and colleagues subsequently expanded the tumor cohort and identified subclasses within the ER-positive group [165].

These individual molecular subgroups are associated with predicted mutations, distinct immunohistochemical profiles and clinical features (Figure 4).

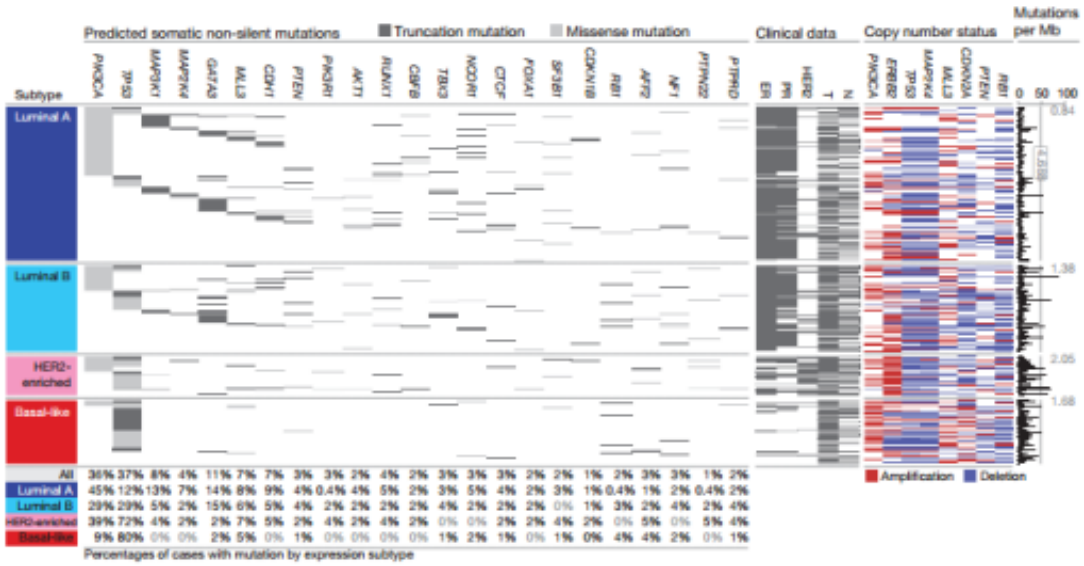

Figure 4. Most frequently mutated genes in breast cancer molecular subtypes and correlations with clinical features. *From [177]*.

According to the aforementioned molecular studies, breast carcinomas can be categorized in the following subtypes:

- ER-positive:
  - Luminal A
  - Luminal B
- ER-negative:
  - HER-2
  - Basal/ Triple-negative
  - Normal breast-like

Luminal A

This subgroup is characterized by hormone receptor positivity (ER and PR), HER-2 negative status and a low proliferation index and represents of 50-60% of diagnosed invasive carcinomas [178]. They are also positive for luminal cytokeratins (CKs), such as CKs 8/18 and present upregulation of certain estrogen receptor related genes (GATA3, FIXA1 and LIV1) [101]. These tumors are usually well-differentiated, of histological low grade and associated with a better prognosis, lower relapse rates and improved overall survival in comparison with other subtypes [179-183]. Given that this type of tumors presents a high expression of hormone receptors, they are usually treated with endocrine therapy or aromatase inhibitors [101, 102].

Luminal B

Luminal B tumors constitute 10-20% of invasive breast carcinomas, and include HER-2 positive tumors. In comparison with Luminal A they present a more variable degree of hormone receptor positivity and a higher cell proliferation rate, as shown by the upregulation of proliferation associated genes, such as CCNB1, MYBL2 and MKI67 [165, 184]. They are commonly of higher grade and biologically more aggressive, exhibiting a worse prognosis with higher chances of relapse than Luminal A tumors [179, 182, 183, 185]. The inclusion of the Ki-67 labeling index brought significant clinical value to the identification of luminal subgroups with poorer prognosis [186].

HER-2

These comprise, approximately, 15% of invasive breast carcinomas and are characterized by HER-2 gene amplification or HER2 protein overexpression [187]. However, part of HER-2 tumors are not clinically HER-2 positive, with patients being treated according to clinical testing results [188]. Additionally, about 40% of tumors in this subgroup present p53 mutations [102]. Consequently, HER-2 positive tumors are

traditionally associated with an aggressive behavior, multifocal/ multicentric disease and extensive nodal involvement [37]. However, the outcome for these tumors has been improved due to the introduction of anti-HER-2 agents in chemotherapy regimens, such as trastuzumab and lapatinib [189-191].

Basal/ Triple-negative

Basal-like tumors present a distinct phenotype, lacking ER, PR and HER-2 expression, but displaying overexpression of basal/ myoepithelial cytokeratins (CKs 5/6, 14 and 17) [192]. Approximately 75% of basal-like tumors exhibit p53 gene mutations [165] and increased EGFR expression [101]. They are more frequent in patients with BRCA1 mutations and particularly of younger age [193]. Histologically, they are generally of high-grade and proliferation index presenting with pushing borders and peritumoral lymphocytic infiltrate. Comparing with luminal tumors, triple-negative carcinomas tend to have a poorer prognosis, with a significantly shorter relapse-free survival, and highly variable response rates to chemotherapy [194]. Treatment for this type of breast tumors remains an active area of research, with the introduction of Poly (ADP-ribose) polymerase (PARP) inhibitors [195, 196] and anti-angiogenesis agents [197, 198] showing promising results.

Main therapeutic strategies for the management of breast cancer involve surgery, chemotherapy and radiotherapy. The type and eventual combination of treatments should be decided and provided by a multidisciplinary team specialized in breast cancer [199, 200].

1.1.3. Breast Tissue Microenvironment

1.1.3.1. Normal Breast Microenvironment

The mammary gland is constituted by several cellular types that work in synchrony by engaging in complex heterotypic communications with neighbour cells and the underlying ECM resulting in a coordinated normal development and function. The microenvironment surrounds the bilayered epithelial ducts and is composed by extracellular matrix and stromal cells, including adipocytes, fibroblasts, myofibroblasts, endothelial cells and several types of leukocytes [201-204].

The mammary gland is a particular organ once part of its development occurs post-natally, more specifically during puberty and pregnancy. Branching morphogenesis refers to the complex developmental program that results in ductal infiltration into the

surrounding stroma as a response to hormonal stimulus [202]. Several studies brought evidence on the fact that cell differentiation and branching architecture is influenced by the tissue microenvironment. Cells grown on plastic culture dishes are not able to differentiate into epithelial cells capable of milk production while cells cultured in three-dimensional reconstituted membrane are [205]. Grafting embryonic salivary gland in adult mammary gland tissue or mammary gland epithelium in salivary stroma results in changed epithelial architecture [206, 207]. Additionally, mammary gland tumor co-culture with embryonic mammary gland stroma leads to tumor differentiation and similar growth to controls [208, 209]. Moreover, a series of studies have demonstrated that culturing tissues from other origin in mammary gland microenvironment causes differentiation into functioning mammary epithelial cells organized in ducts [210-212].

In this context, myoepithelial cells are of particular importance given that they not only influence the differentiation, polarity and proliferation of epithelial cells, as they also, in combination with the basement membrane, constitute a physical barrier between epithelial and stromal cell niches [213, 214]. Signaling through essential basement membrane components, such as laminin-322 and collagen IV are essential for branching architecture given that genetic knockout (KO) of collagen IV receptor (integrin α2β1) subunits blunts branching morphogenesis during pregnancy [215-217]. In turn, laminins are essential for tissue-specific differentiation through several mechanisms, such as polarity definition or cytoskeleton reorganization [202, 218].

Adipocytes constitute one of the microenvironment central players, as they contribute to vascularization, epithelial cell proliferation and leukocyte recruitment through the release of several growth factors and chemokines, besides providing a frame for branching epithelia support [219, 220].

Fibroblasts are also key elements in the mammary gland tissue microenvironment. Its primary function is to participate in the deposition of the collagen-enriched ECM underlying the mammary gland ducts [221, 222]. As other tissue microenvironment cell types, fibroblasts modulate branching morphogenesis by producing factors that act on epithelial cells. Hepatocyte growth factor (HGF) is of particular importance, given that it influences epithelial cell proliferation, migration and branching by activating PI3K signaling [223-226]. Fibroblast growth factor 2 (FGF2) is also necessary for the branching of mammary epithelial cells and has also been shown to promote angiogenesis and consequently contribute to a proper oxygen and nutrient supply [227-229].

In addition to the above mentioned players, several types of leukocytes are also necessary for a correct mammary gland development. For example, macrophages that

are recruited to the tissue stroma in response to epithelial cell colony stimulating factor-1 (CSF-1) production, not only clear shed epithelial cells from the ducts during lumen formation, but also contribute to the arrangement of fibrillar type I collagen, terminal end bud geometry and side branching [230-232]. Similarly, mast cells and eosinophils, recruited through eotaxin secretion, also participate in branching morphogenesis [232, 233]. These studies point to the presence of immune system cells as a normal aspect of a functional mammary gland tissue. However, recent studies provide evidence that alterations in the number and profile of tumor infiltrating immune cells is associated with poor outcome [234].

1.1.3.2. Breast Tumor Microenvironment

Invasive breast tumors lie in a complex microenvironment composed of an altered ECM and several stromal cell populations, most of which increase in cell number during the carcinogenic process. In fact, not only cell numbers increase in general as all breast tissue cell types present a transformed pattern of gene expression during cancer progression [235-239]. The importance of the microenvironment for invasion has been clearly demonstrated in a study by Ma and colleagues revealing that while 5900 genes are differentially expressed in epithelial cells between normal and DCIS, only three genes are differentially expressed between DCIS and invasive carcinoma [236]. Although pathologists have long noticed that certain histopathological characteristics, such as leukocyte infiltration or fibrosis present prognostic value, only more recently evidences have been provided suggesting that tissue microenvironment heterogeneity contributes to the typical features of breast cancer molecular subtypes [240-243]. Actually, changes in the tissue microenvironment may occur even earlier that at the DCIS stage, with epithelial cell signaling resulting in the secretion of chemokines that cause the accumulation of leukocytes, fibroblasts, myofibroblasts, mesenchymal and endothelial cells (Figure 5) [235, 236, 244-246].

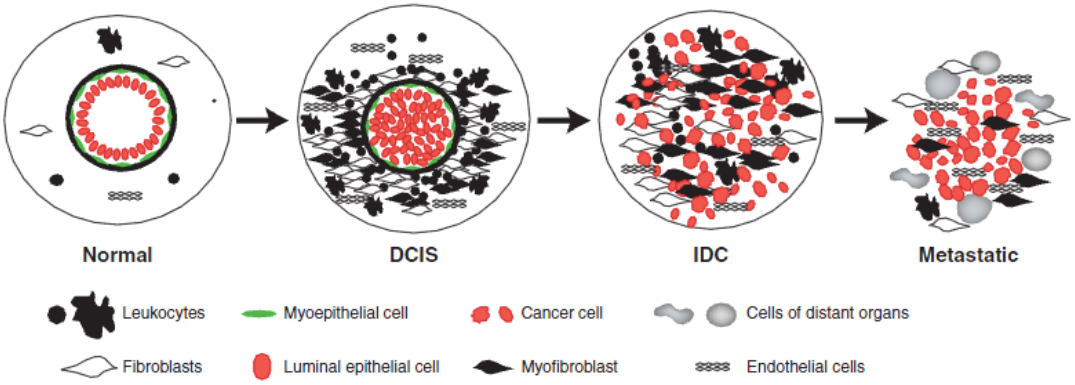

Figure 5. Alterations in the tissue microenvironment during breast cancer progression. ***From [201].***

Increased expression of genes associated with invasion and angiogenesis is also observed in myoepithelial cells from DCIS, revealing the potential role of these alterations in the collapse of the basement membrane. In this sense, myoepithelial cells function as "natural tumor supressors" by maintaining the physical basement membrane barrier that is lost during invasion [214, 247-249]. Furthermore, the expression genes associated with myoepithelial cell differentiation (smooth muscle actin [SMA], oxytocin receptor [OXTR]) is also lost or downregulated in DCIS [235].The lack of clonally selected gene mutations in myoepithelial and stromal cells suggests that epigenetic mechanisms may be the potential drivers of altered expression patterns in these cell populations [203, 250-252].

Subsequent paracrine, autocrine, juxtacrine, chemokine and heterotypic cellular communications govern breast cancer progression, similarly as they guide mammary gland morphogenesis [202]. Malignant epithelial cell signaling drives mesenchymal stem cell and fibroblast differentiation into myofibroblasts that secrete angiogenesis, proliferation and motility promoting growth factors, such as vascular endothelial growth factor (VEGF), epidermal growth factor (EGF) and transforming growth factor β (TGF-β) [253-256].

The complex interactions between cancer cells and leukocytes are often called of "Cancer Immunoediting", and refers to the fact that although the immune system acts as an extrinsic tumor suppressor, it may also be hijacked by the tumor in order to promote its development, invasion and metastasis [257, 258]. The process is characterized by an elimination, equilibrium and escape phases [259], often referred to as the seventh hallmark of cancer [260, 261]. So instead of protecting against carcinogenesis, leukocyte-driven chronic inflammation promotes tumor proliferation, progression and invasion, leading to the release of factors that not only promote the accumulation of genetic

mutations in malignant cells as it also recruits other immune cells that might further be subverted by the tumor [259, 262, 263].

Tumor cells also secrete CSF-1 that attract macrophages to the stroma, than by its turn produce multiple angiogenesis and proliferation factors and pave the stroma for eventual cancer cell metastasis [264-267]. High macrophage infiltration, particularly in areas of necrosis and increased vascular density, is associated with a worse disease-free survival prognosis [265, 268, 269]. Macrophage requirements for both tumor progression and metastasis have been demonstrated in a transgenic mouse model susceptible to mammary cancer with a null mutation in the CSF-1 gene with delayed invasive and metastatic carcinoma. Transgenic expression of CSF-1 restored the invasion and metastasis process, and was associated with an increased infiltration of macrophages in the primary tumor [270]. Macrophage activity is also controlled by other immune system cells, with helper T-cells, through interleukin (IL)-4 secretion, playing a particularly preponderant role in macrophage polarization towards the tumor-associated macrophage (TAM) phenotype [271-274]. In turn, monocytic myeloid derived suppressor cells (MDSCs), through an elevated expression of arginase 1 (Arg1) and inducible nitric oxide synthase (iNOS), non-specifically halt T-cell function and proliferation while recruiting regulatory T-cells (Tregs) to the tumor microenvironment [275, 276]. Macrophages are thought to facilitate tumor progression by two mechanisms:

- Macrophages promote the migration of breast epithelial cells towards blood vessels by a macrophage-tumor cell feedback loop. Malignant breast epithelial cells produce CSF-1 that attract colony stimulating factor 1 receptor (CSFR)-positive macrophages, than in its turn secrete EGF, promoting the proliferation of EGFR-positive malignant cells [267, 277, 278]. IL-6 also participates in breast cancer cell-MDSC paracrine signaling. IL-6 expression by malignant breast epithelial cells recruits MDSCs to the primary tumor and preferential metastatic niches. Conversely, these MDSCs also secrete IL-6 and IL-6 receptor (R) subunit α, promoting a positive feedback loop of increased tumor microenvironment IL-6 [279].
- Perivascular macrophages in the invasive front are also in close proximity to tumor vessels, and this type of tissue arrangement predicts distant metastasis independently of lymph node status [280]. Recently, a study has suggested that this process may be triggered by direct physical contact between macrophages and tumor cells with consequent formation of RhoA-dependent invadopodia and migration [281].

Macrophages probably constitute the tumor microenvironment cell type most associated with tumor progression [268, 282, 283]. Mahmoud *et al.* demonstrated that high numbers of cluster of differentiation (CD)68-positive macrophages is associated with worst breast cancer-specific survival [282]. On the other hand, overexpression of the macrophage migration inhibitory factor (MIF) was associated with an overall better prognosis [284].

Tregs further contribute to the tumor microenvironment immunosuppression. They are selected by the production of reactive oxygen species (ROS) and other inflammatory factors that also assist their immunosuppressive activity by disturbing the redox balance of other immune cells [285-288]. In primary tumors and lung metastasis the frequency of glycan-binding protein galectin-1 (Gal1) positive cells was found to be positively correlated with Treg numbers and upregulated by transforming growth factor-β (TGF-β). Stromal and tumor cell secreted Gal1 binds to the surface glycoproteins of other immune cells and shifts them towards an immunosuppressive phenotype [289]. Other barriers preventing a functional lymphocyte cancer cell recognition and tumor elimination include the sequestration of tumor antigens and major histocompatibility complex (MHC) molecules and loss of costimulatory factors needed for a proper cytotoxic CD8+ T-cell activity [290-292]. A decreased number of CD4+ Th1 T-cells or their precursors, resulting in a skewing of the normal Th1/Th2 ratio and decreased cooperation with cytotoxic CD8+ T-cells may also contribute to the failure to suppress tumor growth [293, 294]. B-cell presence is also frequent in breast tumors as part of the humoral immune response. Recent studies have shown that increased number of B-cells represents an independent indicator for survival [295], particularly in triple-negative (TN) breast cancer patients [240]. Additionally, Levy and coworkers also demonstrated that the number of natural killer (NK) cells may predict recurrence in patients with early stage breast cancer [296].

The invasion of epithelial cells by basement membrane breach and the accumulation of leukocytes and cancer-associated fibroblasts (CAFs) results in a dramatic remodeling of the ECM. The ECM functions as an intermediate in signaling communication between the several cell types, and in turn, these cells can regulate both its composition and structure [297-299]. ECM remodeling through synthesis, degradation, alignment and cross-linking of the matrix [6, 297, 300, 301] affects signaling, potentially leading to tumor cell proliferation and migration, angiogenesis and inflammation [222, 302-304]. Collagen I alignment and stiffening in the tumor microenvironment not only promotes signaling communication between cells as it also sets trails that aid epithelial and stromal cells migration [305-308], and represents an independent negative prognostic

factor for disease-free survival [309].This ECM rearrangement is promoted by cross-linking enzymes, such as lysyl oxidase, whose expression is modulated by hypoxia inducible factor 1-α (HIF1-α) suggesting an alternative route by which hypoxia within breast tumors may promote metastasis [310-312]. Besides its function as a physical scaffold, collagen I also has the ability to modulate numerous signaling pathways [313, 314], for example, by clustering integrins or regulating SNAIL1 stability to enhance extracellular signal-regulated kinase (ERK) activation [315, 316].

CAFs form an important cellular component of the tumor microenvironment, arising from local fibroblasts and their bone marrow precursors through microenvironment production of TGF-β and platelet-derived growth factor (PDGF) [317-319]. Besides collagen I, CAFs also secrete muscle actin fibers and proteoglycan that aid in the tumor microenvironment reorganization in order to promote tumor outgrowth, growth factor storage, and disruption of the healthy surrounding tissue [320, 321]. Hypoxia is also directly related to tumor microenvironment rearrangement through CAFs: CAFs secrete VEGF and FGF2 as a response to cell membrane mediated Notch receptor-ligand signaling in the presence of HIF1-α [322, 323]. New blood vessels represent not only a source of oxygen and nutrients but also a path for the recruitment of more CAFs, following C-X-C chemokine ligand 12 (CXCL12) secretion into the blood stream. Additionally, CAF secretion of CXCL14 into the bloodstream also promotes the recruitment of TAMs, MDSCs and Tregs to the tumor microenvironment [323]. On the other hand, Notch signaling by CAFs induces p53 expression in normal fibroblasts, facilitating tumor infiltration due to an increased antimitogenic response of cells capable of restoring the ECM [324]. Along with several factors that influence cancer cell proliferation, motility and metastasis, such as insulin growth factor (IGF), matrix metaloproteinase (MMP) 2 or chemokine (C-C motif) ligand (CCL)5 [323], CAFs production of FGF2 and fibroblast growth factor receptor 2 (FGFR2) promotes an even more aggressive, hormone independent, tumor behavior by constitutively activating PRs [325].

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1.2. Iron Homeostasis in Breast Cancer

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Mini-review

Iron homeostasis in breast cancer

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ABSTRACT

Iron is an essential element and a critical component of molecules involved in energy production, cell cycle and intermediate metabolism. However, the same characteristic chemistry that makes it so biologically versatile may lead to iron-associated toxicity as a consequence of increased oxidative stress. The fact that free iron accumulates with age and generates ROS led to the hypothesis that it could be involved in the etiogenesis of several chronic diseases. Iron has been consistently linked to carcinogenesis, either through persistent failure in the redox balance or due to its critical role in cellular proliferation. Several reports have given evidence that alterations in the import, export and storage of cellular iron may contribute to breast cancer development, behavior and recurrence. In this review, we summarize the basic mechanisms of systemic and cellular iron regulation and highlight the findings that link their deregulation with breast cancer. To conclude, progresses in iron chelation therapy in breast cancer, as a tool to fight chemotherapy resistance, are also reviewed.

1. Preface

Iron is an essential trace element and the most abundant transition metal in the human body. Due to its ability to accept and donate electrons, while conversion amid ferric (Fe^{3+}) and ferrous (Fe^{2+}) oxidation states, iron is a critical component of sensor, transporter and storing molecules and enzymes involved in energy production and intermediate metabolism [1,2]. Iron is also vital for the cell division process once the enzyme responsible for the synthesis of deoxyribonucleotides, ribonucleotide reductase (RR), is iron dependent [3]. Iron presence is imperative for the R2 RR function, and a limitation of iron availability for R2 leads to loss of RR activity [4,5] resulting in G1/S phase arrest. Iron availability was also shown to regulate other proteins implicated in cell cycle modulation and DNA damage sensing, such as Mdm2, GADD45 and p21/WAF1 [6–9]. Moreover, iron is a functional component of heme and iron-sulfur cluster-containing proteins synthesized in mitochondria [10]. Iron is, thus, fundamental for cell survival, growth and differentiation [11].

On the other side, iron associated toxicity may occur, as a consequence of its strong Lewis acidity and multiple valence [12]. This characteristic aspect of iron chemistry contributes to the formation of hazardous molecules through Fenton and Haber–Weiss reactions. These are classical iron-catalyzed redox reactions that result in the production of hydroxyl radicals and anions in the presence of hydrogen peroxide [13–15]. Consequently, an excess in the cell's labile iron can result in increased oxidative stress and damage in the DNA, lipids and proteins [16–19].

The cell's constant need for iron is challenging in a way that the organism must acquire enough iron for all biological processes where it is needed, while avoiding free iron toxicity [20,21]. Although almost all organisms possess the adequate mechanisms to regulate iron acquisition and maintain its homeostasis, there has been growing body of evidence linking a deregulation of iron homeostasis and a number of diseases, such as cancer, inflammatory and neurodegenerative diseases [22].

Breast cancer is the most common type of cancer in women worldwide [23] and despite recent advances many tumors become chemo-resistant, requiring new strategies for disease control. Recently, several groups have attempted to link a deregulation of iron's metabolism with breast cancer progression, aggressiveness and recurrence.

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2. Introduction

2.1. Systemic iron homeostasis and regulation

Given the fact that there is no physiologically regulated pathway for iron elimination its regulation is achieved through absorption, utilization, storage and export [24].

Generally, iron is obtained through the diet, which is composed by inorganic non-heme iron (±10%), mostly in vegetables, and heme iron (±90%) in meat. Erythropoiesis iron source comes from recycling of senescent macrophages from the reticuloendothelial system [1].

Iron reaches blood circulation through the apical and basolateral membranes of the enterocyte [25]. Inorganic non-heme iron is imported through DMT1 (divalent metal transporter 1), after reduction of its Fe^{3+} form, most likely, by the reductase DcytB (Duodenal cytochrome B), whose expression is induced by iron deficiency and is localized in the apical membrane of intestinal enterocytes [26]. Nevertheless, DcytB does not seems to be the only ferrireductase at the apical membrane of enterocytes, since DCYTB (Cybrd1−/−) knockout mice do not develop iron malabsorption [27,28]. DMT1 belongs to the Nramp family of transmembrane-segment proteins [29–31] and was recognized as essential through defects in iron absorption and assimilation by erythroid precursor cells in mice with microcytic anemia (mk) and Belgrade (b) rats, who share a unique spontaneous mutation in DMT1 (G185R) [30,32]. DMT1 is regulated at the transcriptional level and at its subcellular localization by iron availability [29,30,33]. Heme iron is putatively absorbed by HCP1 (heme carrier protein 1) [34,35]. Although this membranar protein is associated with the greatest heme iron absorption, its mechanism remains unknown because the transporter appears to carry mostly folate [25]. Some heme iron might follow a different trail and bypass enterocytes through Bcrp/Abcg2 and/or Feline Leukemia Virus C Receptor (FLVCR) [34,36,37]. Iron inside the enterocyte is then either stored inside ferritin or transported across the basolateral membrane to be exported into circulation, by the only iron exporter identified to date, ferroportin 1 (FPN1). The iron stored inside enterocyte's ferritin is never used due to enterocyte sloughing into the gut lumen [20]. FPN1 is most likely the only iron exporter, given that gene targeted inactivation of FPN1 is embryonic lethal in Slc40a1−/− mice [38]. Iron export at the basolateral membrane also depends on hephaestin ferroxidase activity, for Fe^{3+} incorporation into transferrin (Tf), and on the multicopper oxidase ceruloplasmin (Cp) [39–42]. The peptide regulatory hormone hepcidin was shown to play a critical role not only in iron absorption, but ultimately, in iron homeostasis through its ability to down-modulate the cellular expression of FPN1 [43–46]. The bioactive hepcidin is a 25 amino acid peptide derived from an 84 amino acid prepropeptide by furin cleavage [47]. Hepcidin is mainly produced by hepatocytes and circulates in blood bound to α2-macroglobulin [48] and its expression is regulated by iron stores, hypoxia, inflammation and rate of erythropoiesis [20,49,50].

2.2. Cellular iron homeostasis and regulation

Once in circulation, iron is incorporated into Tf. Tf function is to transport iron in the bloodstream and deliver it in iron requiring organs, while keeping it nonreactive in the circulation and extravascular fluids [11]. Cellular iron uptake is primarily regulated by the presence of the ubiquitous membrane protein transferrin receptor 1 (TfR1). Tf binding to TfR1 forms a complex that induces receptor mediated endocytosis and internalization of the clathrin-coated endosome [51–54]. A proton pump mediated decrease in the pH occurs (pH ∼ 5.5) facilitating iron release due to conformational changes in the Tf–TfR1 complex [55,56]. Fe^{3+} is then reduced to Fe^{2+} by the endosomal ferrireductase six-transmembrane epithelial antigen of the prostate 3 (Steap3) [57] and DMT1 transports it to the cytosol [29,30,32,58]. A TfR1 homolog, TfR2, is also capable of holo-transferrin binding and internalization [59]. However, this does not appear to be its primary function since TfR2 knockout mice are capable of iron storage and accumulation [60]. It is thought that the HFE gene product, a MHC class I-type protein, in conjunction with β2-microglobulin associates with either TfR1 or TfR2 and senses transferrin saturation, to consequently regulate hepcidin expression [61–63].

In cytoplasm, iron enters the 'labile iron pool' (LIP), and from there it can be used in the production of iron–sulfur (Fe–S) containing proteins or it can be stored in ferritin [64,65]. Ferritin is an ubiquitous heteropolymer consisting of 24 heavy (H) and light (L) subunits that are encoded by different genes. These chains assemble into a shell-like structure capable of storing up to 4500 Fe^{3+} in form of ferric oxy-hydroxide phosphate [64,66,67]. Ferritin is essential for health, as shown by the embryonic lethality of H-ferritin knockout [68]. Iron entrance to ferritin appears to be aided by poly(rC)-binding protein 1 (PCBP1) [69]. H-ferritin has ferroxidase activity, necessary for the incorporation of iron into holo-ferritin, and L-ferritin functions as a nucleation centre. Iron deposits may also be found inside haemosiderin, an iron storage-complex composed by ferritin degradation products [70].

In order to finely control iron uptake, storage and export, sensing of cellular iron content is achieved by a post-transcriptional mechanism operated by the iron-regulated RNA binding proteins 1 and 2 (IRP1 and IRP2) that interact with conserved iron responsive elements (IREs) in the untranslated regions of central components in the iron homeostasis system in vertebrates [71–77]. In iron-depleted cells, IRPs are capable of high affinity binding to their target IREs. The interaction between IRPs and the five IRE copies in TfR1 3′-UTR mRNA stabilizes it, by inhibiting nuclease digestion, and favors its translation [71]. Conversely, IRP binding to the IRE in the 5′-UTR of Ft mRNA leads to a steric blockade in the translation initiation complex, and therefore to decreased protein abundance, in order to enhance metal availability [78]. In conditions of high cellular iron content, IRP1 and IRP2 are inactivated and cannot bind IREs. This leads to TfR1 mRNA degradation and Ft mRNA translation. This reaction inhibits further iron uptake by transferrin, while promoting storage of excess cellular iron, capable of mediating chemical reactions involving the production of reactive oxygen species (reviewed in [20,70,73,79]). The recognition of the existence of putative IREs in other mRNAs has increased the complexity of the IRE-IRP system, while unraveling new functions beyond the regulation of iron uptake and storage [75,80]. These include IREs in 5′-UTR of ferroportin [81,82], aminolevulinic acid synthase (ALAS2) [83], mitochondrial aconitase (ACO2) [84], succinate dehydrogenase [85], glycolyate oxidase (GOX) [86] and hypoxia-inducible factor 2α (HIF2α) [87,88] mRNAs, and in the 3′-UTR of DMT1 [29], cell division cycle 14 homolog A (CDC14A) [89], hydroxyacid oxidase 1 (HAO1) [90] and CDC42 binding protein kinase α/MRCKa (CDC42BPA) [91] mRNAs. The presence of IREs in several mRNAs, identified in a transcriptome-wide approach, suggests that IRP control extends well beyond cellular iron regulation [92,93].

3. Cancer cell iron metabolism

Deregulation of the molecular mechanisms of iron absorption, storage, use and removal can result in disease [75,94,95]. The observation that iron tends to accumulate in the elderly and that free iron in excess is prone to chemical reactions involved in the generation of ROS, led to the hypothesis that iron could be involved

in the pathogenesis of several chronic diseases [96–98]. A few pathways have been proposed for iron-related carcinogenesis. Experimental data indicate that iron induced oxidative stress may cause DNA, protein and organelle damage, through production of hydroxyl radicals and hydrogen peroxide via Haber–Weiss and Fenton-type reactions [16,99,100]. Furthermore, it is conceived that chronic failure in the redox balance has the potential to modulate specific signaling networks associated with malignancy [101], while leading to loss of tumor suppressors [102] and oncogene expression [103]. On the other hand, given iron's critical role in cell proliferation, it is reasonable to assume that iron is also important in the clonal expansion of malignant cells [104,105], besides conferring selective advantage and increased tumor proliferation. Cancer cells seem to present an iron-deficient phenotype with an increased expression of iron importers and decreased expression of iron exporters [106–108].

Some examples of iron metabolism related proteins alterations in cancer cells are presented next:

(a) *Transferrin.* Besides the hepatocytes in the liver, other cell types, like lymphocytes and Sertoli cells also synthesize apo-Tf for specific tissue proliferation and differentiation [109–111]. Certain cancer cell types also secrete Tf-like factors, which could favor cell proliferation and tumor growth in poorly vascularized areas, by acting as an autocrine growth factor [112–114].

(b) *Transferrin Receptor 1.* Probably the most striking evidence for neoplasic cells' need for iron is the massive upregulation of TfR1 in several cancer cell types [115–121]. The studies by Trowbridge et al. highlighted the importance of iron for cancer cell proliferation by showing that anti-TfR1 monoclonal antibodies were capable of growth inhibition in a T leukemic cell line *in vitro* [120]. These results provide strong evidence of iron's pivotal role in cancer cell proliferation, accounted by the high rate of proliferation and DNA synthesis [122–124].

(c) *Receptor-mediated endocytosis.* Aside from Tf-bound uptake of Fe, some normal and neoplastic cells also appear to internalize Fe from low molecular weight (LMW) Fe complexes, such as Fe-citrate and Fenitriacetate [125–130]. Although the biological significance of this Fe-uptake pathway is not fully understood, some suggest that it may represent a mechanism of tumor cell's iron withdrawal by damaged cells [111].

(d) *TfR2.* TfR2 has been found to be expressed in several cell lines and tumors [59,131–133]. Studies on the effect of desferrioxamine (DFO) on the proliferation of Chinese Hamster Ovary (CHO) cells demonstrated that the iron chelator was less effective in cells expressing TfR2 and that TfR2-expressing cells were able to develop into tumors in nude mice, while CHO control cells were not [59].

(e) *Ceruloplasmin.* It is traditionally viewed as a promoter of cellular iron release, due to its ferroxidase activity [134]. Nevertheless, some authors found that ceruloplasmin expression, instead, stimulated iron intake in iron-deficient HepG2 cells [135].

(f) *Ferritin.* Although cancer patients normally do not present elevated iron stores, their serum ferritin is increased in a number of neoplasias [136–138]. Reports about cellular ferritin are quite inconsistent for different types of cancer. Tumor cells normally contain lower ferritin levels than their normal counterparts [139], though some tumors [140,141], particularly, neuroblastomas show high ferritin amounts [142,143]. Once H-ferritin sequesters excess iron, its downregulation may increase intracellular iron availability [144] and favor the suppression of mechanisms involved in immunological responses [145,146]. Work done by Wu and colleagues showed that the H-ferritin and IRP2 are transcriptional targets for repression by c-MYC and that H-ferritin downregulation was essential for transformation via c-MYC [144]. In opposition, Modjtahedi and coworkers showed that c-myc transfection in a SW 613-S human colon carcinoma cell line resulted in H-ferritin overexpression [147]. It is possible that these contradictory results mimic what happens *in vivo*, and H-ferritin modulation by c-MYC is dependent on the cell type.

(g) *IRPs.* Overexpression of IRP1 on tumor growth was evaluated in IRP1-constitutively expressing H1299 lung cancer cells. IRP1-transfected cells did not proliferate more than IRP-wild type cells. Actually, IRP1 expression seems to suppress the growth of xenografts in nude mice. [148]. Another set of experiments with the same type of cell line, demonstrated that IRP2 overexpression results in the stimulation of tumor xenograft growth [149]. Moreover, IRP2 knockdown was able to induce apoptosis and suppress breast cancer growth in a xenograft model [150]. In general, these results appear to suggest that IRP1 may act as a tumor suppressor, whereas IRP2 as an oncogene [70,151]. This hypothesis is further highlighted by studies demonstrating a correlation between IRP2 activity and extracellular-signal regulated kinase 1/2 (ERK1/2) increased phosphorylation and c-MYC expression [144,149].

(h) *Hepcidin.* Recent reports also suggest a role for hepcidin involvement in tumor progression, which can be explained mainly through the action on its downstream target, ferroportin. Hepcidin has been described to be overexpressed in increasing stages of colorectal [152] and renal cell [153] carcinomas, potentially representing a novel oncogenic signaling pathway. On other hand, studies on hepatocelular carcinoma have been consistent on demonstrating hepcidin mRNA supression in cancerous tissues, but not in their normal counterparts [154,155], independently of ferroportin and TfR2 expression [154]. Given hepatocyte's role in hepcidin production, it is possible that malignant transformation may be associated with the disruption of iron sensing through TfR2 and BMP/Smad pathway.

4. Iron metabolism and breast cancer

The argument that iron may promote the development of breast cancer is supported by epidemiological data showing controversial associations between intake of iron-rich diets and breast cancer risk and animal studies that have consistently demonstrated that iron-rich diets or iron injected subcutaneously favors breast cancer progression at several stages. Besides iron's classical role as a critical element for cell proliferation and co-carcinogenic behavior, there is another pathway by which it can favor breast carcinogenesis. Estrogen is a steroid hormone that affects the growth, differentiation and function of several tissues, such as the breast, skin and bone [156]. Estradiol metabolism is a known key step in breast cancer development [157]. Redox cycling of catecholestrogen metabolites generates oxygen radicals [158]. Studies demonstrated that estrogen metabolites in incubation with purified rat liver cytochrome P450 reductase and NADPH form superoxide and reduce ferritin-bound Fe^{3+} to Fe^{2+}, causing potentially reactive iron release [159]. Current knowledge suggests that iron and estrogen may act synergistically. Iron data from the Third National Health and Nutrition Examination Survey (NHANHESIII) demonstrated that parallel and inverse changes occur between estrogen and iron levels during the menopause switch [160]. While estrogen levels decrease as a result of ovarian function's ceasing, iron levels tend to increase due to the suspension of menstrual periods [160,161]. Although

serum estrogen levels decrease after menopause, 17β-estradiol concentrations in breast tissue do not significantly differ between pre- and post-menopausal women, due to cyclooxygenase type II (COX-2) overexpression and consequent increased prostanglandin E2 production, which stimulates estrogen biosynthesis [162,163]. This association between estrogen and iron has been proposed to be a major modulator of breast cancer aggressiveness and recurrence differences between pre- and postmenopausal women [164]. Studies conducted in Huang's laboratory hypothesize that iron deficiency due to menstruation periods in premenopausal women stabilize hypoxia-inducible factor-1α (HIF-1α) in the breast and consequently increase vascular endothelial growth (VEGF) expression. On the other hand, menstrual period cessation and higher iron levels in breast tissue leads to higher incidence rates via the oxidative stress pathway [165,166]. Recently, another Xi Huang's coworker has published a study where they report a functional estrogen responsive element (ERE) upstream the transcription start site of the hepcidin gene, responsible for hepcidin mRNA reduction in *in vitro* HepG2 cells and *in vivo* in mice, after estradiol treatment. These results add evidence to a mechanism by which estrogens may influence not only breast tissue iron levels, but also systemic iron homeostasis, through hepcidin [167,168]. Furthermore, it was shown that E2 and iron exert additive and synergistic effects on ER+ cell lines, with increased Ki67 and proliferating cell nuclear antigen (PCNA) [169]. E2 also upregulates TfR1 [169], and Tf expression, possibly, through an estrogen-responsive element in the promoter region of the Tf gene [170]. Estrogen and iron activate oxidative stress pathways, via ROS production, which induce and maintain the oncogenic phenotype of cancer [171]. It is thus possible that combined effects may result in increased ROS production and site-specific or random DNA damage. Site-specific DNA damage evidence has been reported in experiments where iron was able to replace zinc in zinc fingers of the DNA-binding domain of the estrogen receptor, *in vitro* and *in vivo*. The proposed 'iron finger' in the presence of hydrogen peroxide and ascorbate generates highly reactive free radicals and a cleavage pattern in the estrogen response element [172].

Although iron has been consistently associated with several key signaling pathways [144,173,174] a link with signaling pathways commonly related with breast carcinogenesis, other than estrogen receptor-associated signaling, such as EGFR/ErbB/HER or BRCA1 have not been established yet. However, there is an emerging body of evidence for other major oncogenic signaling pathways. For instance, Wnt signaling, which has been for long implicated in iron-associated alterations in neoplasia, mainly through c-myc overexpression driven H-ferritin downregulation and transferrin receptor 1 upregulation [106,175–177]. The β-catenin activation of cyclin D1, Jun and CD44 [178] are good targets for breast cancer due to their role in cell proliferation, differentiation and stem-cell renewal [179]. Nevertheless, it seems like this pathway might be more relevant in post-menopausal breast cancer since current work in Xi Huang's laboratory revealed that iron deficiency (related to premenopausal breast cancer) mainly altered Notch, but not TGF-β and Wnt signaling in primary breast tumors, driving the activation of epithelial-to-mesenchymal (EMT) transition. This was evident by an increased expression of Snai1 and expected decreased expression of E-cadherin [180]. Iron was also implicated in the control of cell cycle progression, via PI3K/Akt signaling in MCF-7 cells. Iron-saturated lactoferrin can stimulate S phase cell cycle entry, requiring Akt activation, and consequent phosphorylation of the G1-checkpoint Cdk inhibitors, $p21^{CIP/WAF1}$ and $p27^{kip1}$. Conversely, treatment of cells with a PI3K inhibitor blocked iron-saturated lactoferrin driven cell cycling. Since retinoblastoma protein phosphorylation was enhanced in the presence of iron-saturated lactoferrin, the authors concluded that it may act as a potential antagonist of Cdk inhibitors and aid E2F during the progression to S phase via PI3K/Akt signaling [181]. In general, a crosstalk between signaling pathways cannot be excluded, and is probable that iron might play multiple roles in this respect.

4.1. Breast cancer cell iron metabolism

The malignant state is characterized by a deregulation in cellular iron homeostasis, particularly by differences in the expression of iron-regulatory proteins. And breast cancer is no exception, with several studies reporting protein expression changes that may help explain breast cancer cells' iron-deficient phenotype. Perhaps the most explored iron-related protein in this context is ferritin. Serum ferritin has been associated with breast cancer risk and recurrence [136,182]. Concerning breast tissue ferritin content, classical studies from Weinstein, Guner and Elliot state that tissue ferritin levels are significantly higher in cancer specimens, and that they are correlated with increasing staging and associated with higher degrees of epithelial proliferation and pleomorphism [141,183,184]. Follow-up data from patients whose breast ferritin concentrations at the time of surgery were known, was able to stratify patients at higher risk of recurrence based on high tissue ferritin concentration [185]. Based on this, Guner and colleagues proposed that breast tissue ferritin would be a more valuable diagnostic marker than serum ferritin. More recent data from ferritin content and ferritin heavy-chain mRNA levels in nipple aspirate fluids have added evidence that ferritin deregulation is associated with the progression of breast cancer toward a more aggressive phenotype [186,187]. Nonetheless, it seems that ferritin high content in breast cancer tissue is more of a result of ferritin overexpression in stromal inflammatory cells than in neoplastic epithelial cells. Rossiello and coworkers described the immunohistochemical ferritin staining mainly in connective stroma and macrophagic cells surrounding the neoplastic tissue and seldomly in malignant epithelial cells [188]. Although a specific connection between ferritin expression and nuclear factor-κB (NF-κB) signaling has not been established for cancer cells it is probable that ferritin upregulation through tumor necrosis factor (TNF) in inflammatory cells may affect its survival in the tumor microenvironment [189]. Pioneer studies done by Maria de Sousa group [190,191] and a latter by Alkhateeb and coworkers in breast cancer cells [192] show that stromal inflammatory cells, specifically lymphocytes and macrophages, are capable of ferritin secretion, particularly in response to pro-inflammatory cytokines, and that extracellular ferritin stimulates the proliferation of breast cancer cells lines independently of its iron content. These results and the fact that molecular and cellular changes in epithelial tumor cells can stimulate the immune process and consequently result in a local inflammatory response [193,194] bring evidence to ferritin as a critical role player not only in epithelial carcinogenic changes but also as an immunosuppressive effector [195,196].

Jezequel and coworkers also described ferritin light-chain expression in tumor-associated macrophages with an M2-like phenotype and validated it as a prognostic biomarker in node-negative breast cancer patients [197]. Similarly to ferritin content, two other studies also measured the levels of metals in breast tissue and found that iron was significantly accumulated in cancer samples [198,199], and that this association was confined to postmenopausal women, which reflects iron accumulation with increasing age and menstrual periods arrest [199]. Transferrin is the major iron binding-protein and an essential growth factor involved in the growth and differentiation of human tissues [119,200,201]. Transferrin staining was first described by Mason and Taylor in periductular cells [202]. After that, transferrin was described to be produced by myoepithelial cells in normal ducts or around neoplastic ducts in carcinomas in situ [188]. Transferrin secretion in breast epithelial cells is stimulated by 17β-estradiol and may be

a supplementary growth factor conferring selective advantage to rapidly proliferating cells [112]. This is in accordance with the significant higher numbers of transferrin receptors observed in pre-malignant and invasive carcinoma lesions [203–208], whose levels are a consequence of cancer cells' elevated iron requirements [17,111,209]. A more recent study suggests that TfR1 may also function in anti-apoptotic and breast cancer survival signaling by Src phosphorylation of TfR1 at Tyr^{20} [210]. Furthermore, the level of transferrin receptor mRNA was associated with poorly differentiated tumors [187] and by immunohistochemistry, with a lower percentage of estrogen receptor-positive cells [203], demonstrating a differential expression in tumors with a more aggressive phenotype.

The first critical evidence that the iron efflux mechanisms were also altered in breast cancer cells was demonstrated by Torti and co-workers. These authors described a marked decrease in the levels of ferroportin, both in breast cancer tissue and cancer cell lines with a high malignant potential. Furthermore, this decrease in protein abundance was associated with a higher LIP concentration and an upregulation of hepcidin, which coincides with the proposed post-translational mechanism of ferroportin degradation by hepcidin. The analysis of tumors from 251 breast cancer patients according to their molecular subtypes also revealed that ferroportin expression was lower in poor-prognosis subtypes (basal, ERBB2+ and luminal B) than in good-prognosis subtypes (normal and luminalA), and that these differences were highly significant ($p < 0.0001$). A low ferroportin expression was also associated with classical indicators of poor outcome, such as high histological grade, absence of estrogen receptor and lymph-node involvement [108]. In a latter work by the same group, an attempt to characterize iron's deregulation in breast malignant phenotype and evaluate these changes' impact on prognosis resulted on a model with 16 genes, which they termed the 'iron regulatory gene signature' (IRGS), capable of stratifying homogeneously treated patients, including node-positive and -negative ER+ patients and patients exhibiting favorable molecular subtypes (normal and luminalA). They noticed that almost 50% of the 61 iron-related genes assessed presented an association with breast cancer prognosis. As suggested by the authors, an integrated prognostic model combining the IRGS and luminal subtypes could provide an increased prognostic value. Gene dyads associated with minimized lower intracellular iron (low-TFRC[transferrin receptor]/high-HFE and high-SLC40A1[ferroportin]/low-HAMP[hepcidin]) further contributed additively to the prediction of low metastatic risk [211]. Given IRP2 role in the regulation of cellular iron homeostasis and since IRP2 knockdown was shown to increase H-ferritin expression and decrease TfR1 expression in triple negative MDA-MB-231 human breast cancer cells, as well as reducing its growth in a mouse mammary fat pad, it is reasonable to argue that most iron metabolism alterations may be driven by IRP2 upregulation. However, it seems that at least for the ERBB2+ subtype another mechanism of iron regulation may be controlling these changes, once IRP2 it is not upregulated in this molecular subtype [150].

Research performed in breast cancer cell lines are in agreement with what is described for primary tumors, with low levels of ferritin (light and heavy chain), transferrin, transferrin receptor 1 and iron regulatory proteins 1 and 2 in cell lines with an epithelial phenotype, contrasting with significantly high levels in cells lines with an aggressive mesenchymal phenotype [206,212]. Shpyleva and coworkers also described an alteration in the subcellular distribution of ferritins. Given that ferritin-heavy chain can translocate to the nucleus to protect DNA from iron-mediated toxicity [67], it is possible that these results point to a critical defense mechanism in cancer cells against iron-induced damage [212]. Based on a series of primary breast carcinomas and a panel of *in vitro* models, a study conducted by Habashy was able to define TfR1 as an independent prognostic biomarker in a subgroup of ER+/luminal-like breast cancer patients characterized by poor outcome and resistance to tamoxifen. CD71 expression was also associated with tumor proliferation, basal cytokeratins (CKs 14 and 5/6), p53, EGFR and HER2. Moreover, the finding that Faslodex and a phosphoinositide-3 kinase (PI3K) inhibitor could partially restrain the Tf-induced growth of MC7-cells suggests an interplay between Tf/CD71 mitogenic signaling, ER and PI3K [206].

Overall, it is conceivable that the deregulation of mechanisms essential to controlled iron homeostasis may contribute to tumor development, more aggressive behavior, metastization and high disease recurrence [111,164]. In conclusion, these results start to shed light on the molecular pathways involved on iron's classical view as a co-carcinogenic element promoting breast tumorigenesis [209].

4.2. Animal models

In animal experiments, iron has been shown to enhance the breast carcinogenic process. Studies in rats have demonstrated that excess dietary iron intake increases the incidence of 1-methyl-1-nitrosourea (MNU) mammary tumors [213,214] and that mammary tumor incidence and burden were lower in Fe-deficient DMBA (7,12-Dimethylbenz(a)anthracene)-treated Sprague Dawley (SD) rats, when compared with iron replete treated rats [215]. These studies, and particularly the one from Diwan and coworkers, where rats received iron (III) sulfate subcutaneously (sc) after DMBA initiation, seem to be consistent on presenting Fe as strong promoting effector of mammary carcinogenesis, as shown by the shortened latency period for tumor appearance and increased tumor incidence and size, when compared with DMBA-only treated animals [216]. Work from Stevens et al. has also suggested that dietary iron can affect the course of mammary and colon carcinogenesis in HFE-mutated individuals, through accumulation of oxidative stress products [217]. Xenotransplantation of mammary tumors into animal models also added evidence that iron nutrition of the host may affect tumor growth, since mice given a low iron diet [218] and rats on a low iron diet either with or without desferoxamine treatment [219] had tumors with lower mean size. However, the greatest inhibitory effect on tumor growth was observed for the combined effect of a low iron diet with the iron chelator treatment [219].

On the other hand, studies by Hardman [220] and Gonzalez [221] present a shift in the paradigm of iron supplementation effect on mammary tumor development by presenting results where supplementation of a high fish oil diet with a prooxidant, such as ferric citrate, suppresses breast cancer growth in xenotransplanted athymic nude mice. The rationale for this hypothesis comes from observations that iron supplementation of high-content fat diets increases lipid peroxidation product levels, with resulting tumor growth suppression [220,221] mitotic index diminution and tumor cell death enhancement [220]. Nevertheless, a more recent study with MDA-MB 231 xenotransplanted mice has shown that not only the iron chelator desferal does not inhibit doxorubicin anti-tumor activity, as it has its own inhibitory effect. Moreover, the anti-proliferation effect, caused by iron depletion, was not observed in normal epithelial cells [222]. It is highly important to clarify this situation once iron nutrition of the host may affect mammary tumor growth. The knowledge should then be applied when delineating personalized treatment regimens.

4.3. Genetic association studies

Considering that HFE mutations are highly prevalent in the general population [223] and that they have the ability to modify iron stores, it is likely that eventual procarcinogenic effects of these

mutations might affect the development, behavior and aggressiveness of tumors. Several studies brought evidence on the fact that elevated iron stores are associated with increased risk for the development of cancer [96,224–226], with one from Zacharski and collegues [227] showing clear evidence of decreased cancer incidence in a group of patients with arterial disease on phlebotomy. Taking into account the biological rationale, it is possible that HFE mutations may affect the prevalence or behavior of breast cancer. However, studies have been inconsistent in proving an association between HFE major alleles and breast cancer. Although a study from Nelson et al. [228] reported no increased risk for breast cancer in heterozygous individuals from a community-based cohort, latter ones have described a positive association for individuals carrying the C282Y allele [229,230]. It is also important to note that in the study conducted by Osborne, the presence of H63D alleles was assessed, and C282Y/H63D heterozygotes were not at increased risk for breast cancer [230]. Interestingly, Beckman and coworkers also suggested that an interaction between HFE and TFR alleles may increase the risk for neoplasia, namely multiple myeloma, breast and colorectal cancers. In the case of breast cancer the association was found in women homo- or heterozygous for the C282Y allele in combination with homozygosity for the TFR Ser142 allele. No association was found for each gene separately [231]. A subsequent study found no effect of the hemochromatosis-transferrin receptor system on the incidence of breast cancer in a German population, although a significantly increased frequency of the HFE C282Y allele was observed in breast cancer patients with lymph node involvement [232]. The most traditional explanation for the associations observed lies on the capacity of HFE to modulate body iron stores and the increased iron uptake associated with the C282Y allele [230,233,234]. This explanation, however, may not be satisfactory since most C282Y heterozygotes do not present increased body iron stores [235]. Alternatively, and since HFE is a nonclassical major histocompatibility complex (MHC) protein, whereby individuals with HFE mutations present an impaired class I antigen presentation pathway [236] and altered CD4/CD8 ratio [237], it is possible that a particular HFE genotype might present a selective advantage for the tumor to escape immune surveillance. It is also proposed that this association may reflect the fact that HFE gene is located in one of the most commonly amplified regions of chromosome 6p, which are directly involved in tumor development and progression (HFE mapped to 6p21.3 locus) [238,239].

4.4. Epidemiological studies

Several case-control, cross-sectional and cohort studies have found positive associations between high iron levels, measured as dietary iron intake, plasma iron and ferritin, total iron binding capacity (TIBC) and overall cancer risk and death [225,240–243]. Nonetheless, epidemiological evidences for breast cancer are inconsistent. This may be due to unequal questionnaires evaluating dietary iron intake, either through food or supplements, disregard for genetic polymorphisms that may alter the regulation of iron uptake or even because most of these studies treat pre- and post-menopausal women as one, when pre and post-menopausal breast cancer etiology vary in many aspects [244]. For instance, studies on dietary iron intake in relation to breast cancer development have held positive [245,246], null [247–254] and even negative associations [255,256]. The acknowledgment of a protective effect for iron intake was unexpected even for the latter authors, proposing the non-evaluation of supplementary iron as one of the study's main limitation.

Likewise, heme iron relation to breast cancer risk has also led to conflicting results. Heme iron is highly bioavailable and may present higher carcinogenic potential as compared with plant-based non-heme iron [257]. From the previous reported studies only Kallianpur and colleagues were able to show a positive association between heme iron intake and breast cancer development, where previously there was not one for total dietary iron intake [254]. However, result discrepancies between studies might reflect different assessments of heme iron proportion from meat samples. Red meat is a source of heme iron and on this basis, several other groups have also dedicated themselves to study the particular relation between meat and breast cancer risk. However, this falls beyond the purpose of this review [258,259].

Studies evaluating simultaneously dietary iron intake and body iron stores through plasma ferritin levels have also presented incomparable results [246]. This can be a result of the amount of iron absorbed from the food (heme iron is more readily absorbed than non-heme iron), phlebotomy frequency and menstrual status [260,261]. This inconsistency might even suggest different roles for dietary iron and metabolically available iron stored as ferritin on the development of breast cancer. Nevertheless, serum and plasma ferritin as a measure of body iron stores have been more consistent on showing results supporting an association with breast cancer risk [182,262,263] and development of nonproliferative fibrocystic changes [246]. It is thus plausible, to argue that iron stores are probably a better marker of mammary tissue exposure to iron than total or heme iron consumption [246]. Although serum ferritin has been long considered the best indicator of iron stores [264,265], several other studies using other measures, such as transferrin saturation, plasma iron and total iron binding capacity, have also assessed possible associations with breast cancer risk, once again with conflicting results [224,243,251,256,266–268]. One study, even examined toenail levels of iron and other trace elements, with an odds ratio of 0.89 (95% CI 0.56–1.40) for breast cancer risk. In addition, factors such as age, time since menopause, use of postmenopausal hormones, body mass index, iron supplement use and alcohol consumption are critical determinants of body iron stores, and particularly of plasma ferritin concentrations in postmenopausal women [269]. Another study published in the same year has shown that the contribution of genetic variation between women prevails over the effects of interindividual variation to body iron stores [270]. Cade and coworkers have also found that the effect of HFE genotype on ferritin serum concentrations occurred, mainly, after menopause [271]. These results, and the long known fact that a significant number of premenopausal women are iron deficient [272,273] brings evidence to the fact that epidemiological studies assessing breast cancer risk in relation to body iron stores' measures should consider pre- and postmenopausal women separately.

5. Iron-chelators in breast cancer therapy

Cancer cells increased dependency on iron, as shown by TfR1 upregulation and increased Fe uptake [115–119] led to the hypothesis that depleting iron from neoplastic cells could represent an approach to limit proliferation and growth. Indeed, several monoclonal antibodies (mAbs) against TfR1 have shown to inhibit transferrin binding [121], cause S-phase arrest in leukemic cells [120] and diminish lymphoma tumor growth in mice [274]. Likewise, antisense TfR1 oligonucleotides also induced TfR1 mRNA reduction, and selective inhibition of breast and hepatoma cells [275,276]. Although breast cancer cells treatment with TfR1 mAbs upregulated the hypoxia pathway, addition of ascorbate at physiologic concentrations was able to restore prolyl hydroxylase (PHD) activity, resulting on HIF1-α downregulation, without prejudice of proliferation suppression [277]. Consequently, drugs limiting iron bioavailability, such as iron chelators, may further inhibit cellular proliferation and represent a new approach to fight chemotherapy-resistance.

5.1. Signalling pathways affected

Initially, Fe chelators inhibitory effects were thought to be solely due to RR inhibition, with consequent cell cycle arrest at G1/S phase due to Fe deprivation [278–280]. However, recent studies propose the involvement of multiple targets and pathways [278]. Genes found to be affected by Fe chelators include the upregulation of p53 [281,282], p21 [8], HIF1-α [282,283] and NDRG1 [284–286] and downregulation of D cyclin [282,287], cdk2 [282] and cdk4 [288]. Particularly in breast cancer, DFO (desferrioxamine) and 311 were found to increase p53 transcriptional activity [289], and desferri-exochelin (D-Exo) was shown to trigger apoptosis in the MCF-7 cell line, while causing reversible cell cycle arrest in normal mammary epithelial cells [290] and HIF stabilization and proaptotic protein NIP3 activation in MDA468 cells [291]. DFO and 311 were also found to decrease $p21^{CIP1/WAF1}$ protein levels, by contributing to proteasomal degradation, and reducing $p21^{CIP1/WAF1}$ mRNA nuclear translocation. The effect appears to be due to Fe depletion, and p53-independent, since it also occurred in p53-mutated cell lines [8,292]. Since approximately 50% of human tumors present mutated p53 [293], the ability to tackle cells with mutant or absent p53 is an advantageous feature [12,123]. Overall, iron chelators seem to act beyond the simple depletion of cellular iron stores, by targeting multiple molecular pathways that affect tumor survival, proliferation and metastasis.

5.2. Synthetic iron chelators

Desferrioxamine – desferrioxamine (DFO) is a hexadentate siderophore excreted by *Streptomyces pilosus* that became the first iron chelator for the treatment of iron overload disorders, such as β-thalassemia [294,295]. DFO has shown anti-proliferative activity against several tumor types [15,296–299]. However, its short-half life, which requires subcutaneous infusion for extensive periods, and high hydrophilicity [65,300], limit its efficacy in clinical practice. *In vitro* and *in vivo* studies in breast cancer xenografted animals confirmed that desferrioxamine inhibits breast tumor growth, through iron depletion [219,222,301,302].

Tachpyridine – tachpyridine is a hexadentate ligand, which consists of three pyridine molecules linked to triaminocyclohexane [12,303]. Although its primary cytotoxicity mechanism is based on Fe chelation, tachpyridine also demonstrated ability to bind Ca(II), Mg(II), Cu(II) and Zn(II) [303–305]. The multiplicity of target binding may explain tachpyridine-mediated cell cycle arrest at G2, when most Fe chelators arrest at G1-S [15]. Tachpyridine has been shown to deplete the iron regulatory pools of breast, non-small cell lung [306] and bladder [304] cells. Tachpyridine also presented ferritin synthesis inhibition [304] and p53-independent induced apoptosis [306].

Aroylhydrazones – the aroylhydrazone class of iron chelators was developed after the discovery of PIH (pyridoxal isonicotinoyl hydrazone)-mediated mitochondrial iron mobilization from reticulocytes, by Ponka et al. [307]. Several studies afterwards have confirmed PIH efficiency as an iron chelator [308–311]. PIH analogues were further synthesized, with proven efficiency for Fe chelation, both *in vitro* and *in vivo* [312–315]. A member of the 300 series, 311, presented the greatest potential in terms of anti-proliferative activity [312,316]. The mechanism by which 311 seems to act on the cells' proliferative capacity, including on breast-derived cell line, seems to be dependent on Fe depletion required for RR activity [279,317]. Recently synthesized salicylaldehyde isonicotinoyl hydrazone (SIH) iron chelator and analogues have flaunt their potential by demonstrating induction of apoptosis and cell cycle arrest in MCF-7 breast cancer cells, while presenting greater selectivity towards cancer cells and improved hydrolytic stability [318].

Thiosemicarbazones – anti-proliferative efficiency of the thiosemicabazone class of iron chelators has been consistently demonstrated in several studies [319–324]. This appears to be due to RR inhibition by Fe binding at its di-Fe centre [319–322] and on the affinity for other transition metals [323,325]. This led to the development of a strong RR inhibitor, 3-aminopyridine carbalhdehyde thiosemicarbazone (Triapine) [326,327]. Over the last few years, Triapine has been enrolled in quite a few Phase I and II clinical trials [328–332], with a currently undergoing Phase II trial on the effectiveness of combining triapine and gemcitabine in patients with refractory metastatic breast cancer [333]. Although the positive results pose Triapine as a potential anti-cancer drug, some adverse effects bring concerns over its toxicity [65,123,300].

Di-2-pyridylketone thiosemicarbazone (DpT) series – The identification of specific structural characteristics for the development of an efficient anti-neoplastic agent pointed to the synthesis of the DpT series [334,335]. From the seven analogues synthesized, Dp44mT exhibited the highest anti-proliferative activity *in vitro*, and displayed greater efficiency than DFO and 311 against several cell lines, including MCF-7 and MCF-7/VP breast cancer cells [286,334]. It proved to be highly selective, since the proliferation of the MRC-5 normal fibroblast cell line was not affected [334]. In addition to its selective killing, Dp44mT has proven to induce DNA double-strand breaks and inhibit top2A (DNA topoisomerase IIA) in MDA-MB-231 cells [336]. *In vitro* results were confirmed *in vivo* by demonstrating that Dp44mT was able to inhibit human tumor xenografts growth in nude mice, with no obvious hematological alterations [286,334]. Its marked anti-proliferative activity is dependent on iron binding [334] and enhanced by the capacity to generate ROS and subsequent oxidative stress [335].

2-Benzoylpyryridine thiosemicarbazone (BpT) series – The addition of a phenyl ring in the place of a 2-pyridyl group preceded the generation of 2-benzoylpyridine thiosemicarbazone (BpT) chelators. These were proposed as being the most effective, selective and less toxic chelators yet [337,338]. The most probable explanation for their high anti-proliferative efficiency lies on the enhanced redox activity [337]. Although *in vivo* results have been promising, further investigation in *in vivo* models is needed [123,300].

5.3. Naturally ocurring iron chelators

Some naturally occurring iron chelators have also some promising effects on breast cancer treatment. Mimosine, a toxic non-protein amino-acid found in the foliage and seeds of *Leucaena glauca* or *Mimosa pudica* [339], has shown to block cell cycle progression in MDA-MB-453 human breast cancer cells, through iron depletion, in a lower dosage than DFO [340]. In the last years, the interest on the phenolic extract of the turmeric Indian spice (diferuloymethane) has also increased, due to its potential as an anti-cancer agent [341,342]. Action mechanisms include NF-kB, COX and p-AKT pathway inhibition [300,342]. Furthermore, anti-angiogenic activity in *in vitro* [343–345] and *in vivo* studies [344] was demonstrated. In breast cancer cell lines, curcumin proved to lower HIF-1α and HIF-2α protein accumulation in hypoxia and to impact negatively in clonogenic cell survival [346]. Although there is some controversy over curcumin's strength as a Fe chelator, its potentially therapeutic effects have rendered its entrance in several clinical trials [300,341].

6. Conclusions and perspectives

Alterations in the basic systemic and cellular mechanisms of iron regulation may cause an imbalance and ultimately result in disease. In the particular case of cancer cells, upregulation of iron importers and downregulation of iron exporters and storing

molecules may rather increase the bioavailable iron, while favoring the tumor cells' proliferation and survival. It is now fairly settled that breast cancer epithelial cells present an iron-deficient phenotype as a result of differences in the expression of iron homeostasis regulatory proteins, and that these may explain the iron's effect on tumor development, behavior and recurrence. The entrance of several iron chelators on clinical trials (either alone or chemotherapy-associated) seems highly promising, but the fact that we still do not know whether the deregulation in the iron metabolism network of proteins is a cause or a consequence of malignancy deserves a more in-depth look. Furthermore, these iron metabolism alterations are not dissected for the breast cancer progression timeline. Leukocyte infiltration is a rather common event in breast cancer, with macrophage and T-cell abundance presenting prognostic value for recurrence-free and overall survival. Macrophages and lymphocytes also play major roles in systemic iron regulation through iron recycling of senescent erythrocytes [20] and non-transferrin bound iron uptake [347], respectively. Given the fact that both macrophages and lymphocytes are capable of ferritin secretion [191,192] and hepcidin production [348–350] in inflammatory conditions it is of mounting importance to the define the 'iron-profile' of these cells in the breast microenvinronment to better assess the value of iron-related therapy.

Conflict of Interest

The authors declare that they have no conflict of interest.

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1.3. Iron Regulation and the Immune System

Iron is a critical element in life, as a fundamental component of several proteins involved in cell cycle regulation and energy production [1, 2]. Iron homeostasis is tightly regulated at the systemic and cellular levels to avoid free iron-associated toxicity [3, 4]. Current evidence suggests that cells of the immune system participate in the systemic and local regulation of iron homeostasis. This is particularly important given the fact that most pathogenic agents are iron-dependent and the host must modulate iron availability in order to limit its use by microorganisms while assuring the body's vital iron needs [5-7]. On the other hand, there are various ways by which iron levels can fine-tune the immune system, evidence supported by the observation that several proteins involved in the regulation of iron homeostasis also display immunological properties [8-17].

The average human male adult contains, approximately, 4 g of iron, of which, more or less, 2.5 g is incorporated in the hemoglobin of erythrocytes. By recycling the iron from senescent erythrocytes and delivering it to erythrocyte precursors for hemoglobin synthesis macrophages are recognized as the most relevant leukocytes in iron homeostasis. They are the main players in iron exchanges to the plasma, by favoring or limiting iron export in response to erythropoietic needs or infection/ inflammation [18, 19]. The aging alterations in erythrocytes that may lead to macrophage recognition comprise Band 3 alterations (which is the most abundant erythrocyte membrane protein) [20, 21], phosphatidylserine exposure in the outer cell membrane [22] and increased membrane rigidity [23]. Following red blood cells phagocytosis, heme is catabolized inside macrophages through the action of heme-oxygenase (HO) – 1, releasing iron into the phagosomal lumen that may subsequently be transported to the cytosol by the divalent metal transporter (DMT) natural resistance-associated macrophage protein (Nramp) 1 [24-26] and then exported through ferroportin (FPN) 1 (Figure 6) [18, 27]. Alternatively, when in excess, heme may be directly exported via feline leukemia virus subgroup C receptor (FLVCR) into the circulation [28].

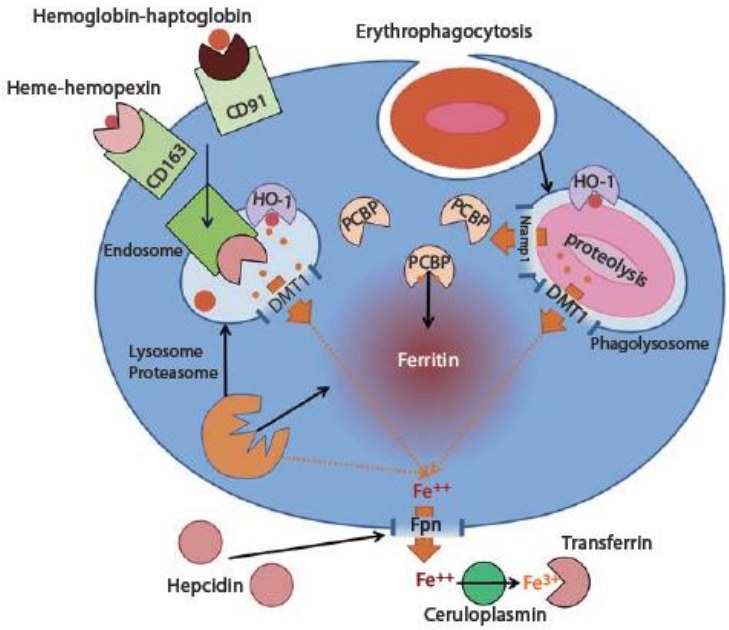

Figure 6. Iron flow in the macrophage. ***From [29].***

Macrophages also regulate plasma iron by sequestering it inside during infection, as a response to augmented IL-6 that increases hepcidin production, and then by its turn degrades FPN1 at the macrophage cell membrane in an autocrine or paracrine manner [30-32]. Given that 90-95% of serum iron is exported by macrophages through the process described above, this constitutes an important process to reduce iron availability to proliferating pathogens while promoting the activity of innate immune cells [33]. In this sense, neutrophils also play a role in decreasing iron availability to invading microbes by secreting lactoferrin [34], an iron binder related to transferrin [35], and lipocalin-2, that binds siderophores, small organic iron chelators used by pathogens to carry iron from the environment [36].

However, prolonged activation of these mechanisms may result in the so called anemia of inflammation or chronic disease, due to limited iron supply for erythropoietic precursors [37-39].

Once macrophages are critical to the modulation iron homeostasis, it may be expected that, on the other hand, iron levels also have the ability to modulate the macrophages' phenotype. Several attempts have been made in order to classify macrophage into subsets. The most successful classification separates macrophages into two 'end-of-the-spectrum' polarization phenotypes participating in particular immunological responses: (1) M1, a subset involved in the responses of type I helper (Th1) lymphocytes to pathogenic agents activated by IFN (Interferon)-γ and Toll-like receptors (TLRs) and characterized by a high expression of MHC class II, IL-12 and tumor necrosis factor (TNF) α and (2) alternatively-activated or M2, that participate in Th2- type responses, such as humoral immunity and wound healing, triggered by cytokines as IL-4 and IL-13 [40, 41]. Recent evidences highlight that differences between M1 and M2 macrophages may surpass their role in inflammation or scavenging [42] and also reflect the way they handle iron. Functionally, iron retention in M1 macrophages from the reticuloendothelial system is a well characterized reaction to inflammation and key to their bacteriostatic role [43, 44]. Macrophage iron accumulation, by itself promotes the proinflammatory activity and contributes to the maintenance of inflammation, without resolution [45]. Sindrilaru and colleagues demonstrated that macrophage iron overloading induced an unrestrained proinflammatory M1 activation state, which was perpetuated through the release of TNFα and hydroxyl radicals. Treatment of an experimental mouse model with iron-dextran resulted in iron accumulation of macrophages presenting a persistent proinflammatory M1 status within the dermis that could be abrogated by treatment with the iron chelator desferrioxamine (DFO) [46]. Conversely, M2 macrophages present an iron exporter phenotype, which is consistent with their functional role as scavengers and wound healing participants [47, 48]. Once tumor-associated macrophages (TAMs) present a similar phenotype to M2 macrophages is expected that this might represent a mechanism of iron supply to a growing tumors [42]. In fact, studies by Recalcati and colleagues demonstrated that M2-conditioned media enhanced the proliferation of tumoral and non-tumoral cell lines [47].

Studies on lymphocyte traffic and positioning set the foundation for the postulate that cells of the adaptive immunological system, which constitute an important circulating body component, by binding iron could participate in the regulation of tissue iron toxicity by limiting its use by potentially harmful cells, such as bacteria and cancer cells [49-52]. Following this idea, De Sousa and co-workers described the first animal model with spontaneous iron overload, the β2-microglobulin (β2m) deficient mouse. β2m is a protein that associates with the alpha chain of MHC class I molecules and other class-I like molecules, such as CD1a or HFE, necessary for its proper expression and stability at the

cell surface [53]. As a consequence, mice lacking β2m present a severely decreased cell surface expression of MHC-class I molecules, almost no CD8+ T-cells and hepatic parenchyma iron overload [54, 55]. Because β2m does not bind the p.C282Y HFE mutated form, one could argue that HFE deficiency was the cause of the described phenotype, but the fact that treatment with hematopoietic cells attenuated the phenotype and deviated the iron overload to Kupffer cells suggested an additional role for leukocytes in the systemic regulation of iron overload [55, 56]. Following studies in older mice reiterated this idea by demonstrating that β2m deficiency causes higher hepatic iron loading, with concurrent steatosis, than HFE deficiency [57]. The fact that the lack of other β2m-dependent molecules contributes to systemic iron deregulation and tissue iron loading was further established by the demonstration that the pattern of tissue iron loading in the HFE-/-RAG1-/- double knockout mice does not recapitulate the phenotype observed in β2m-/-Rag1-/- double knockout mice, particularly in what refers to the heart and pancreas sparing [59]. Furthermore, δTCR-/- mice, lacking $\gamma\delta^+$ intraepithelial lymphocytes also present increased liver iron accumulation, in comparison with control mice [60].

Besides evidences in animal models, other observations have highlighted that alterations of lymphocyte frequencies and/or of its subpopulations are linked to a deregulation of iron homeostasis. For a long time it has been known that there is a negative correlation between the number of T lymphocytes, particularly CD8+, and the severity of iron overload in hereditary Hemochromatosis (HH) patients [61, 62]. T-cell phenotypical and functional anomalies, either associated with intrinsic iron overload or the HFE p.C282Y variant itself have also been described [9, 63]. Oversaturation of transferrin results in increased iron uptake by lymphocytes with a concomitant decrease in their proliferation, to levels below to what is observed in the absence of transferrin [64]. Conversely, iron deficiency is also critical for T cell differentiation since Transferrin Receptor 1 (TFR1) deficiency results in arrest at the triple negative stage (CD3-CD4-CD8-), while not affecting severely B cell development [65]. Furthermore, *in vitro* B and T-cell proliferation is inhibited by incubation with TFR1 antibodies [66, 67]. Dhur and colleagues demonstrated that anemic mice presented not only a lower proportion of Thy-1+ splenocytes, CD4+ and CD8+ T-lymphocytes in the spleen, but also decreased percentage and absolute number of cell subtypes per spleen. Consistent with this, not only iron deficiency is associated with impaired lymphocyte activation after phytohematogglutinin, concanavilin A and lipopolysaccharide (LPS) stimulation [68, 69] as

[1] RAG1 – is a gene whose expression is restricted to lymphocytes during developmental stages and crucial for the maturation of B and T-lymphocytes (for review see [58] J.M. Jones, M. Gellert, The taming of a transposon: V(D)J recombination and the immune system, Immunol. Rev., 200 (2004) 233-248.)

an alteration of the cytokine profile of the activated lymphocytes may occur, with an increased expression IFN-γ and lower expression of IL-4 [70].

More recently, lymphocytes have also been suggested to attenuate tissue iron toxicity by participating in the uptake of non-transferrin bound iron (NTBI). NTBI refers to iron associated with low molecular weight complexes, responsible for iron-associated oxidative stress and resulting toxicity in iron-overload disorders. Arezes and coworkers have demonstrated that T lymphocytes take up and accumulate iron in a similar form to hepatocytes, supporting their role as buffers against iron-mediated toxicity [71]. In a subsequent study they further cemented this idea by demonstrating that T lymphocyte reconstitution, through adoptive transfer in a T lymphocyte-deficient mouse model submitted to an iron-rich diet prevented liver and pancreas iron accumulation [72].

Overall, these observations confirm the proposed influence of the host iron status in the behavior of the immune system, particularly in infection situations where iron overload may contribute to increased susceptibility to iron-dependent pathogenic agents. On the other hand, the immune system, by chronic immune activation may also modulate iron homeostasis and perpetuate associated immunopathologies.

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Chapter 2

Rationale and Aims

Despite recent advances in the knowledge of basic biology, diagnosis and treatment, breast cancer remains the most common type of cancer in women worldwide, with many becoming chemo-resistant, which demands new strategies for disease control. In the last decade we have witnessed a growing body of evidence linking the imbalance of iron homeostasis with the development, behavior and progression of neoplastic diseases. Particularly, in breast cancer, current data suggests that an iron regulatory gene signature associated with minimized epithelial intracellular iron content may predict a favorable outcome, particularly in ER+ patients treated with tamoxifen monotherapy.

In spite of the established impact of genetic and epigenetic changes in breast epithelial cells in breast cancer progression, it is now well accepted that these are not sufficient for the acquisition of a fully malignant phenotype. In this respect, gaining insight about the mechanisms by which the cells of the microenvironment promote tumorigenesis is of vital importance. The potential role of cells that take part in systemic iron regulation, such as lymphocytes and macrophages, has not been established in the context of local iron homeostasis in the breast.

The main goal of this doctoral thesis was to characterize the iron-associated phenotype of breast epithelial cells, lymphocytes and macrophages in different stages of breast cancer progression. For that purpose, and making use of a series of breast aesthetic reduction specimens, DCIS and IDC, the following studies were performed to assess specific objectives:

I. **Analyze the iron-related phenotype of epithelial cells, lymphocytes and macrophages in human normal breast and carcinoma samples.**

   Using antibodies against Hepcidin, FPN1, TFR1 and Ferritin (FT) the purpose was to characterize the iron-utilization or iron-donor phenotypes of epithelial cells, lymphocytes and macrophages from primary breast cancer samples, and metastized and non-metastized lymph nodes. The potential association between the expression of these iron-related proteins and classical clinicopathological markers of breast cancer behavior and progression was also a specific aim of this study.

II. **Evaluate the role of the chemokine CCL2 as a potential modulator of tissue iron status in breast cancer.**

   We aimed at testing if the expression of CCL2 in breast epithelial cells and macrophages could be a modulator of tissue iron deposition and of the iron-exporter phenotype observed in lymphocytes and macrophages. Moreover, the

association between CCL2 and the clinicopathological markers of breast cancer behavior and progression was also assessed.

III. **Test the influence of the HFE variants, p.C282Y and p.H63D, on the expression of iron-related proteins.**

The previously established expression of the iron-related proteins was further evaluated in relation to the presence of HFE variants, in order to test the hypothesis that these are modulators of the expression of iron-related proteins in breast tissue.

IV. **Analyze the iron-related phenotype of epithelial cells, lymphocytes and macrophages in benign lesions and mammary gland tumors from cats and dogs.**

Using the same approach as for objective I. we aimed at verifying if the cat and dog are good animal models for human breast carcinogenesis, i.e., if they recapitulate or not the variations observed in the expression of iron-related proteins in human breast cancer.

Chapter 3

Local Iron Homeostasis in the Breast Ductal Carcinoma Microenvironment

RESEARCH ARTICLE Open Access

Local iron homeostasis in the breast ductal carcinoma microenvironment

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Abstract

Background: While the deregulation of iron homeostasis in breast epithelial cells is acknowledged, iron-related alterations in stromal inflammatory cells from the tumor microenvironment have not been explored.

Methods: Immunohistochemistry for hepcidin, ferroportin 1 (FPN1), transferrin receptor 1 (TFR1) and ferritin (FT) was performed in primary breast tissues and axillary lymph nodes in order to dissect the iron-profiles of epithelial cells, lymphocytes and macrophages. Furthermore, breast carcinoma core biopsies frozen in optimum cutting temperature (OCT) compound were subjected to imaging flow cytometry to confirm FPN1 expression in the cell types previously evaluated and determine its cellular localization.

Results: We confirm previous results by showing that breast cancer epithelial cells present an 'iron-utilization phenotype' with an increased expression of hepcidin and TFR1, and decreased expression of FT. On the other hand, lymphocytes and macrophages infiltrating primary tumors and from metastized lymph nodes display an 'iron-donor' phenotype, with increased expression of FPN1 and FT, concomitant with an activation profile reflected by a higher expression of TFR1 and hepcidin. A higher percentage of breast carcinomas, compared to control mastectomy samples, present iron accumulation in stromal inflammatory cells, suggesting that these cells may constitute an effective tissue iron reservoir. Additionally, not only the deregulated expression of iron-related proteins in epithelial cells, but also on lymphocytes and macrophages, are associated with clinicopathological markers of breast cancer poor prognosis, such as negative hormone receptor status and tumor size.

Conclusions: The present results reinforce the importance of analyzing the tumor microenvironment in breast cancer, extending the contribution of immune cells to local iron homeostasis in the tumor microenvironment context.

Keywords: Breast cancer, Ferroportin 1, Iron, Stromal inflammatory cells, Tissue microenvironment

Background

Breast cancer ranks as the most frequent neoplasia and cause of cancer death, in spite of growing advances in early diagnosis and novel therapy regimens [1]. A change in this scenario demands a better understanding of the cellular and molecular processes involved in breast cancer development and progression.

As a fundamental element involved in cell metabolism, division and proliferation, iron has been implicated as an important player in cancer development [2]. The argument that iron may promote the development of breast cancer is supported by animal studies consistently demonstrating that iron-rich diets or iron injected subcutaneously favors breast cancer progression at several stages [3–6]. From the cell biology perspective, it is now well accepted that the malignant state in breast epithelial cells is characterized by a deregulation in cellular iron homeostasis, as revealed by differences in the expression of several iron-related proteins relating with markers of poor outcome [7–11]. Particularly, a marked decrease in the levels of the iron exporter ferroportin 1 is observed

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both in breast cancer tissue and cancer cell lines with a higher malignancy potential, denoting the relative "iron-deficient" phenotype compatible with their increased proliferative status [12, 13]. In spite of the known impact of genetic and epigenetic changes of breast epithelial cells in tumor progression, it is acknowledged that these are not sufficient for the acquisition of a fully malignant and invasive potential [14–16]. Stromal inflammatory cells, which are present in the breast tissue even before malignant transformation, may also induce alterations in the breast microenvironment that ultimately can drive tumorigenesis [17, 18]. Pioneering studies by De Sousa and co-workers have shown that lymphocytes and macrophages are capable of synthesizing and secreting ferritin [19, 20]. More recent work by Alkhateeb and co-workers not only confirmed that breast cancer-associated macrophages secrete ferritin, particularly in response to pro-inflammatory cytokines, but also that extracellular ferritin stimulates the proliferation of breast cancer cells [21]. Also, Jezequel and co-workers have described ferritin light-chain expression in tumor-associated macrophages with an M2-like phenotype and validated it as a prognostic biomarker in node-negative breast cancer patients [22]. Of note, in vitro M2 polarized macrophages present an iron-release prone phenotype, with higher transferrin receptor 1 and ferroportin 1 expression than classically activated M1 macrophages, which is thought to contribute to its iron recycling function as scavengers of senescent and apoptotic cells and in tissue remodeling [23, 24]. To our knowledge, the expression of ferroportin 1 in breast cancer tumor-associated lymphocytes and macrophages has never been addressed before.

In the present study we analyzed the iron-profiles of epithelial cells, lymphocytes and macrophages in normal human breast and ductal carcinoma samples and assessed their association with clinicopathological markers of cancer progression and behavior. With this approach we reinforce the evidence that favors the contribution of stromal inflammatory cells to breast tumor microenvironment while highlighting the potential role of lymphocytes and macrophages in the regulation of local iron homeostasis.

Methods

Sample characterization

Selected and stored human breast tissue samples referred for histological analysis at the Pathology Service at Centro Hospitalar do Porto (between 2004 and 2009), were re-analyzed. We selected 131 samples corresponding to 58 cases of invasive ductal carcinomas (IDC), 16 cases of ductal carcinomas in situ (DCIS) and 57 samples without evidence of breast disease obtained from breast reduction aesthetic surgery, as controls. Axillary lymph node samples from 14 non-metastized and 12 metastized IDC were randomly selected from the initial cohort and analyzed. In addition, 6 frozen core biopsy samples, collected in 2013, from patients with invasive ductal carcinomas from which written informed consent was obtained, were selected for imaging flow cytometry studies. Pathological and clinical features, including histological diagnosis, estrogen receptor (ER), progesterone receptor (PR), human epidermal growth factor 2 (HER-2) status and peripheral white blood cell (WBC) count data were available from the interin pathology reports. ER, PR and HER-2 status were assessed by immunohistochemistry, as routinely done in the Pathology Service. HER-2 ambiguous results were confirmed by FISH.

Tissue microarray construction

Formalin-fixed paraffin-embedded (FFPE) tissue blocks and hematoxylin and eosin (H&E)-stained slides were retrieved from the archive and re-evaluated by an experienced pathologist (CL). Representative areas from normal breast epithelium, ductal carcinoma in situ and invasive ductal carcinoma lesions were selected, marked on the H&E slides and then sampled into the tissue microarray (TMA) collector blocks. Most selected lesions corresponded to "pure" DCIS or IDC lesions, i.e., from samples with the corresponding classification. Whenever possible, non-malignant and DCIS lesions were also selected from invasive ductal carcinoma cases. Two tissue cores from human donor liver samples were also included in each tissue microarray block, as positive controls. A total of 452 FFPE 2 mm breast tissue cores were used for the tissue microarray construction from which 405 were assessable. 2 μm-thick TMA sections were cut in a microtome and H&E stained. Histologically, each core was classified by the pathologist without previous knowledge of the type of donor sample. Cores with 'normal' breast histology retrieved from DCIS or IDC samples were further classified as 'normal in DCIS' and 'normal in IDC', respectively. Representative areas with malignant lesions from DCIS and IDC were classified as DCIS "pure lesions" or IDC "pure lesions", respectively. DCIS cores retrieved from IDC samples, without signs of invasion, were classified as DCIS in IDC. The numbers of cores included for each histological type and type of donor sample are summarized in Table 1.

Immunohistochemistry

Immunohistochemical staining was performed in 2 μm-thick TMA sections with the following antibodies: rabbit polyclonal anti-human hepcidin-25 antibody (dilution 1:500, Abcam, Cambridge, UK [25]), rabbit polyclonal anti-human ferroportin 1 antibody (FPN—1:500, Novus

Table 1 Number of spots included in TMA receiver blocks

Tissue sample	Type of core	No. of cores in TMA blocks
Control Normal Samples	Normal	119
DCIS	Normal in DCIS	12
	DCIS pure lesion	54
IDC	Normal in IDC	61
	DCIS in IDC	39
	IDC pure lesion	120

Abbreviations: *TMA* Tissue Microarray, *DCIS* Ductal Carcinoma *In Situ*, *IDC* Invasive Ductal Carcinoma

Biologicals Europe, Cambridge, UK [26]), rabbit polyclonal anti-human ferritin antibody (FT—1:1000, Sigma-Aldrich, MO, USA [27]), mouse monoclonal anti-human CD71 (TFR1 [clone 10 F11]- 1:80, Novocastra, Newcastle, UK [28]), mouse monoclonal anti-human CD68 (clone Kp-1, 1:2000, A. Menarini Diagnostics, CA, USA), mouse monoclonal anti-human CD163 (clone MRQ-26, 1:100, Cell Marque, CA, USA), mouse monoclonal anti-human CD80 (37711, 1:100, R&D Systems, MN, USA), rabbit polyclonal anti-human CD4 (clone H-370, 1:250, Santa Cruz Biotechnology, TX, USA) and mouse monoclonal anti-human CD8 (clone C8/144B, 1:100, Cell Marque, CA, USA). The sections were deparaffinized twice in xylene, rehydrated in decreasing concentrations of ethanol and washed in water. Heat-mediated antigen target retrieval was done with DakoTarget Retrieval Solution (Agilent Technologies, Denmark). Immunohistochemistry was performed according to Novolink Polymer Detection kit procedures (Leica, Biosystems, Cambridge, UK). Enzyme reactivity was visualized using 3,3′-Diaminobenzidine tetrahydrochloride (DAB, Sigma-Aldrich, MO, USA) and slides were counterstained with Mayers hemalum solution (Merck Millipore, Darmstadt, Germany), dehydrated and mounted with Entellan (Merck Millipore, Darmstadt, Germany). The reaction obtained in all samples was observed in a Leica DM LB microscope. Each antibody optimum dilution was determined in a tissue positive control. Slides with replacement of the primary antibody with an antibody of the same immunoglobulin isotype were integrated in each experiment as negative labeling controls. A section of liver tissue from a HAMP (hepcidin) KO mouse was also included as a hepcidin negative control.

Staining criteria

Tissue specimens from normal control, DCIS and IDC samples were immunostained for hepcidin, ferroportin (FPN1), transferrin receptor 1 (TFR1) and ferritin (FT) proteins and their cellular localization examined in epithelial cells (Hepcidin, $n = 323$; FPN1, $n = 315$; TFR1, $n = 308$; FT, $n = 325$), lymphocytes (Hepcidin, $n = 175$; FPN1, $n = 174$; TFR1, $n = 177$; FT, $n = 244$) and macrophages (Hepcidin, $n = 173$; FPN1, $n = 150$; TFR1, $n = 178$; FT, $n = 245$). A semi-quantitative evaluation method was applied as follows: the score obtained by the percentage of positive cells (0 % = 0 points; 1–10 % = 1 point:, 11–20 % = 2 points:, 21–35 % = 3 points:, 36–50 % = 4 points: and >50 % = 5 points) was multiplied by the score obtained by the staining intensity (no staining = 0 points, weak staining = 1 point, moderate staining = 2 points and strong staining = 3 points). We are aware that this type of scoring results in a higher number of area groups. However, we considered that grouping the area percentages in groups with higher intervals would also introduce high variation inside each group. Cores from the same donor tissue diagnosed with the same histological type were grouped and their mean score calculated. Lymph node iron-related proteins immunoexpression assessment was done in B cell and T cell areas and in macrophages. Scores ranged from 0 to 3, where 0 was considered absence of immunoexpression, 1, low expression, 2, moderate expression, and 3, high expression of the correspondent iron-related protein.

Perls' Prussian blue staining

Hemosiderin deposits were detected by the routine technique of Prussian blue histochemical staining. Briefly, after deparaffinization and rehydration in the ethanol series, sections were immersed in a mixture of equal volumes of potassium ferrocyanide solution and hydrochloric acid solution, both at 2 %. Counterstaining was achieved with nuclear fast red (Merck Millipore, Darmstadt, Germany). The absence or presence of hemosiderin deposits was evaluated in epithelial and stromal inflammatory cells.

Imaging flow cytometry

OCT-embedded frozen samples from 6 core biopsies were cut in a cryostat and H&E stained for pathological assessment of malign disease. After thawing, biopsies were gently removed with a scalpel and allowed to mechanically disaggregate with the help of forceps. Cells were resuspended in 2 % BSA (Bovine Serum Albumin, Merck Millipore, Darmstadt, Germany) in PBS, and set for staining in a 96-well standard microplate. A Neubauer counting chamber was used in order to count and stain 1×10^6 cells in every assay. After centrifugation at 2000 rpm and resupension in 0.2 % BSA in PBS, cells were incubated with mouse monoclonal anti-human cytokeratin FITC ([clone 1B3] IOTest, Beckman Coulter, Madrid, Spain), mouse monoclonal anti-human CD68 PE-Cy7 ([clone Y1/82A] eBioscience Affymetrix, CA, USA), mouse monoclonal anti-human CD3 PerCP-Cy5.5 ([clone SK7] BD, Madrid, Spain), rabbit polyclonal anti-human FPN PE (Novus Biologicals Europe, Cambridge,

UK), mouse monoclonal anti-human CD20 PE-Cy7 ([clone B9E9] IOTest, Beckman Coulter, Madrid, Spain) and FPN PE (Novus Biologicals Europe, Cambridge, UK) [staining 2]. Cells were washed with 0.2 % BSA in PBS and centrifuged at 2000 rpm prior to fixation with Fixation Medium from Fix & Perm Cell Fixation and Permeabilization Kit (Life Technologies, CA, USA) and then resuspended in 0.2 % BSA in PBS for analysis. Single-stained and unstained cells were used as controls. Data were acquired in an imaging flow cytometer (ImageStream®, Amnis, EDM Millipore, Darmstadt, Germany) using a 488 nm laser. Images and data were acquired using INSPIRE Software v4.0 (Amnis, EDM Millipore, Darmstadt, Germany). Brightfield was detected on channel 1, FITC on channel 2, PE on channel 3, PerCP-Cy5.5 on channel 5 and PE-Cy7 on channel 6. A total of 100 µL was loaded per sample and 8000 events meeting the cell classifier were acquired at a 40× magnification (image pixel 0.5 μm^2). Compensation and analysis were performed in IDEAS v6.0.348 software (Amnis, EDM Millipore, Darmstadt, Germany). Data was compensated through a matrix created based on the single-stained cell controls. A hierarchical gating strategy was created in the software in order to identify breast epithelial cells, lymphocytes and macrophages. Briefly, first focused cells were selected (gradient root mean square of the brightfield) followed by gating on single-cells (brightfield area *vs* aspect ratios). T-Lymphocytes were then selected on an Intensity_CD3 PerCP-Cy5.5 *vs* Area on Channel 1 plot, B Lymphocytes on an Intensity_CD20 PE-Cy7 *vs* Area on Channel 1 plot, macrophages on an Intensity_CD68 PE-Cy7 *vs* Area on Channel 1 plot and finally epithelial cells on an Intensity_cytokeratin FITC *vs* Area on Channel 1. Gated cells were excluded from further analysis before selecting the next population. FPN1 intensity in the cell membrane and cytoplasm was measured through the creation of masks defining the total area of the cell and then the correspondent cytoplasm by eroding the cell membrane in channel 1 (cell membrane = total cell—cytoplasm).

Laser capture microdissection

Six µm-thick sections from the axillary lymph nodes were cut and placed in PALM® 1.0 polyethylene naphthalate (PEN) membrane slides (Carl Zeiss MicroImaging GmbH, Germany). Before use, slides were treated with UV irradiation at 320 nm for 30 min as recommended by the manufacturer. Immediately prior to microdissection, slides were deparaffinized, rehydrated and stained with Mayers hemalum solution (Merck Millipore, Darmstadt, Germany). Lymphocyte and macrophage exclusive regions in metastized lymph nodes were selected, cut and catapulted into individual PALM® adhesive cap microcentrifuge tubes (Carl Zeiss MicroImaging GmbH, Germany). Microcentrifuge tubes with the areas of interest were transported on ice and RNA was extracted immediately.

RNA extraction and real-time PCR

Isolation of total RNA was performed with the Absolutely RNA FFPE kit (Agilent Technologies, California, USA), according to the manufacturers' protocol. Briefly, sections from each archival sample were deparaffinized and incubated overnight with a lysis buffer containing proteinase K and submitted to a series of washes-on-column until elution. Immediately after, 50 ng of RNA were reversed transcribed with Maxima First Strand cDNA Synthesis Kit (Thermo Fisher Scientific, MA, USA) in a total volume of 20 µL, according to the manufacturer's protocol. Evaluation of FPN1 mRNA levels (Hs00221783_m1) was performed in a Rotor-Gene 6000 instrument (Qiagen, CA, USA) with a TaqMan® Probe-based gene expression assay (Applied Biosystems, CA, USA). Reactions were carried out in triplicate and gene expression levels calculated relative to GUSB mRNA levels (Hs99999908_m1). Mean relative expression was calculated based on the formula ΔCt = Ct target gene—Ct endogenous control gene and fold change on $2^{(\Delta Ct \text{ breast tumor samples} - \Delta Ct \text{ normal breast samples})}$.

Statistical analysis

Sample distributions were compared using Kruskal-Wallis or Mann-Whitney's U tests. Pearson's Chi-Square was used to evaluate the differences between categorical variables. The Spearman's rank correlation coefficient was used to evaluate the relationship between variables. In figures, experimental errors are shown as one standard error of the mean. Data were analyzed in IBM SPSS Statistics 20.0 software and statistical significance was accepted at $p < 0.05$.

Results

Immunolocalization and relative expression of iron-related proteins in breast tissue

Immunolocalization of hepcidin, FPN1, TFR1 and FT was assessed in breast tissue samples of normal controls, DCIS and IDC cases. As seen in the representative images illustrated in Fig. 1, different staining patterns were apparent among sample types and, within samples, among the different cell types. Leukocyte infiltrate was much more pronounced in carcinoma than in normal mastectomy samples. Using the semi-quantitative data (described in Methods) obtained exclusively in representative cores of "pure" lesions, we assessed the expression of hepcidin, FPN1, TFR1 and FT in epithelial cells, lymphocytes and macrophage in normal and cancer (DCIS and IDC) breast tissue. The results are illustrated in Fig. 2.

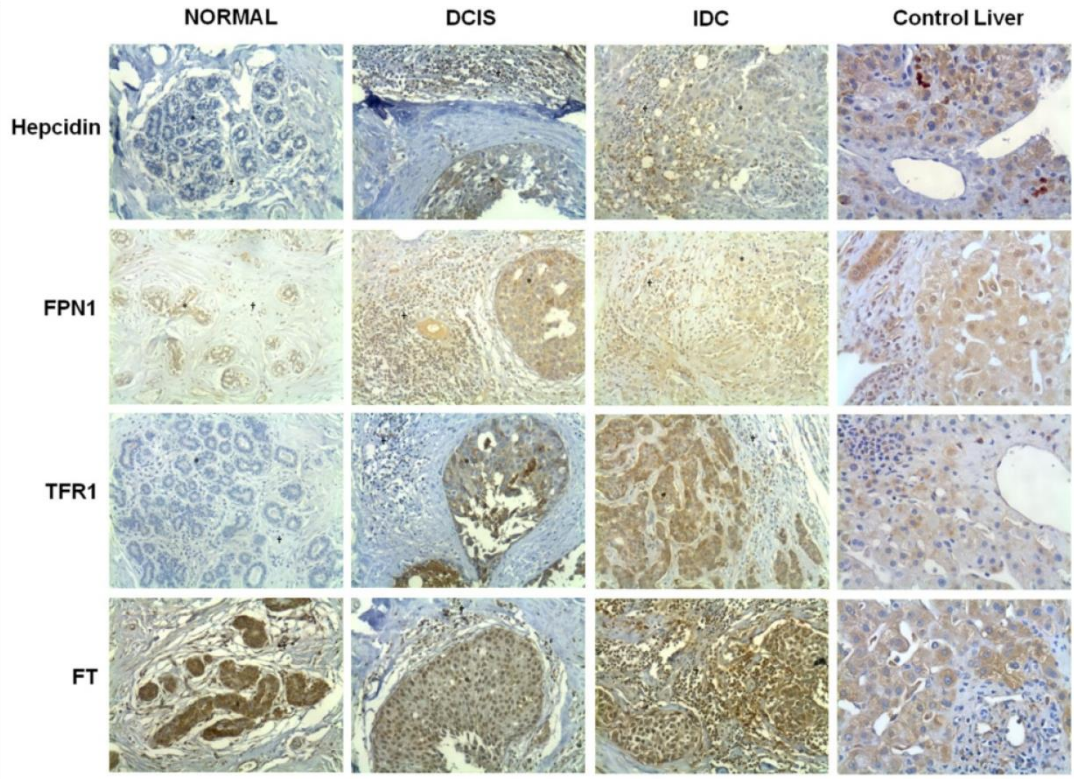

Fig. 1 Hepcidin, FPN1, TFR1 and FT expression pattern in breast tissue. Representative images of Hepcidin, FPN1 (Ferroportin 1), TFR1 (Transferrin Receptor 1) and FT (Ferritin) immunostaining in normal breast, DCIS (ductal carcinoma *in situ*) and IDC (invasive ductal carcinoma). A section of human donor liver is also shown as a positive control. Tissue microarrays containing several samples of breast tissue and human donor liver were constructed, sectioned and subjected to immunohistochemistry, as described in materials and methods (Original magnification × 400). * and † are representative of epithelial cells and leukocyte infiltrate, respectively

Hepcidin

Hepcidin expression was restricted to the cytoplasm in all cell types detected. Breast cancer epithelial cells (in DCIS and IDC) presented a significantly higher expression of hepcidin than in control normal samples ($p < 0.001$) (Fig. 2a). The pattern of differential expression was similar for the stromal inflammatory cells analyzed. Breast cancer infiltrating lymphocytes and macrophages also presented significantly higher expression of hepcidin ($p = 0.002$ and $p < 0.001$, respectively) (Fig. 2b,c).

Ferroportin 1

FPN1 expression in breast epithelial cells was mainly observed in the cytoplasm but, in some cases, also in the cell membrane. In lymphocytes and macrophages it could only be detected in the cytoplasm. Regarding epithelial cells, no significant differences were observed for FPN1 expression between normal samples, DCIS and IDC (Fig. 2a). However, in breast carcinoma samples, lymphocytes and macrophages expressed significantly higher levels of FPN1 than in normal samples ($p = 0.014$ and $p < 0.001$, respectively) (Fig. 2b,c), with FPN1 expression in macrophages being higher in DCIS samples ($p < 0.01$ when compared with IDC samples) (Fig. 2b).

Samples with FPN1-expressing T-lymphocytes are composed by a combination of CD4 and CD8 cells (Fig. 3). Tissue section staining with CD68 (macrophage lineage marker), CD80 (M1-like) and CD163 (M2-like) led to the observation that while in normal samples the macrophage population comprises a combination of comparable numbers of cells expressing CD80 and CD163, in breast carcinoma samples this population is predominantly composed of CD163-positive cells, and thus associated with an M2 (alternative) macrophage polarization phenotype (Fig. 4).

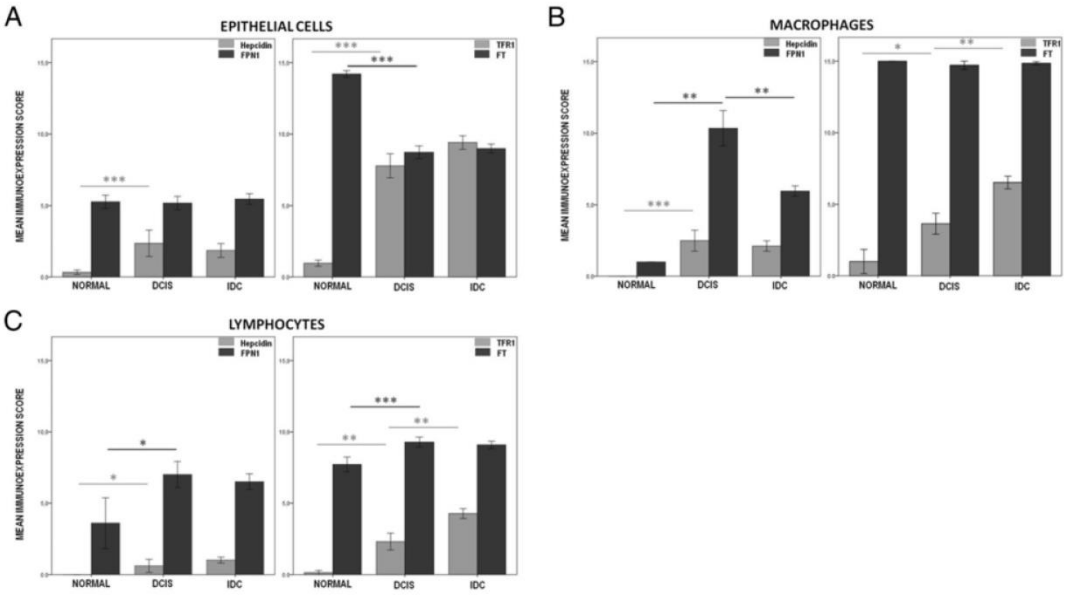

Fig. 2 Hepcidin/FPN1 and TFR1/FT phenotype dyads in breast tissue. A semi-quantitative method of assessing the immunoexpression in the TMA sections was applied by multiplying the area and intensity staining scores, as described in materials and methods. The scores ranged from 0 to 15. Graphs show Mean ± SEM. Significant differences are shown for comparison with the precedent group $*p < 0.05$, $**p < 0.01$, $***p < 0.001$, Mann Whitney's U test; **a** Immunoexpression score for breast epithelial cells in control normal samples, DCIS (ductal carcinomas *in situ*) and IDC (invasive ductal carcinomas) for hepcidin ($n = 121$), FPN1 (Ferroportin 1, $n = 113$), TFR1 (Transferrin Receptor 1, $n = 119$) and FT (Ferritin, $n = 119$); **b** Immunoexpression score for macrophages in control normal samples, DCIS (ductal carcinomas *in situ*) and IDC (invasive ductal carcinomas) for Hepcidin ($n = 75$), FPN1 (Ferroportin 1, $n = 62$), TFR1 (Transferrin Receptor 1, $n = 73$) and FT (Ferritin, $n = 91$); **c** Immunoexpression score for lymphocytes in control normal samples, DCIS (ductal carcinomas *in situ*) and IDC (invasive ductal carcinomas) for Hepcidin ($n = 77$), FPN1 (Ferroportin 1, $n = 70$), TFR1 (Transferrin Receptor 1, $n = 73$) and FT (Ferritin, $n = 91$)

Transferrin receptor 1

TFR1 expression was predominantly detected in the cytoplasm of all the cell types analyzed. Nonetheless, in epithelial cells of some breast carcinoma samples a clear membranar staining was also observed. TFR1 expression was significantly higher in epithelial cells, lymphocytes and macrophages from breast carcinoma samples ($p < 0.001$) in comparison with control normal samples (Fig. 2). Furthermore, TFR1 immunoexpression in infiltrating lymphocytes and macrophages was, as expected, higher in IDC samples ($p < 0.01$) when compared with DCIS (Fig. 2b,c).

Ferritin

FT expression was predominantly observed in the cytoplasm of epithelial cells, lymphocytes and macrophages.

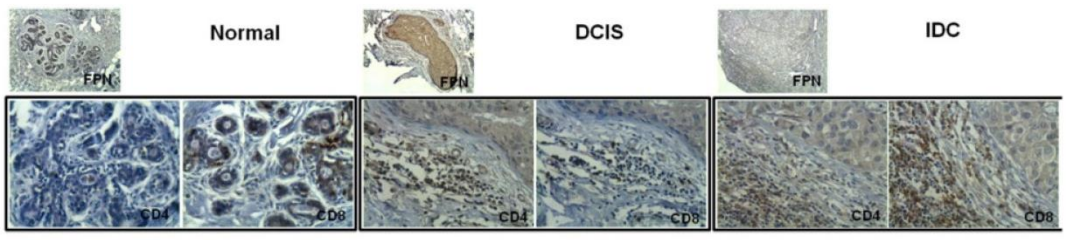

Fig. 3 FPN1-expressing leukocytes are composed by a mixture of CD4 and CD8 T-cells. Sections of normal breast tissue, DCIS (ductal carcinoma *in* situ) and IDC (invasive ductal carcinoma) to reveal the presence of CD4 and CD8 T cells, in FPN1-expressing leukocyte infiltrate. For details see Materials and Methods (Original magnification × 100- upper FPN images, ×400- CD4 and CD8 images)

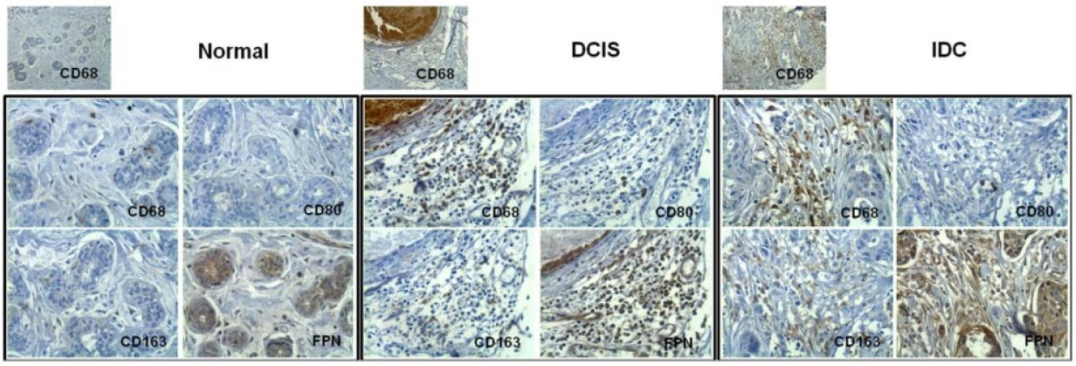

Fig. 4 FPN1-expressing leukocytes in carcinomas are predominantly M2-like. Sections of normal breast tissue, DCIS (ductal carcinoma *in* situ) and IDC (invasive ductal carcinoma) to reveal the presence of cells of the macrophage lineage (CD68), and its classical polarization phenotypes, M1-like (CD80) and M2-like (CD163), in FPN1-expressing leukocyte infiltrate. For details see Materials and Methods (Original magnification × 100- upper CD68 images, ×400- squared image series)

Breast cancer epithelial cells presented a significantly lower expression of FT than normal samples ($p < 0.001$) (Fig. 2a). On the other hand, FT expression in breast cancer infiltrating lymphocytes was significantly higher than in normal samples ($p < 0.001$) (Fig. 2c). No significant differences were found regarding FT in macrophages, given that its expression was consistently high in all the samples analyzed (Fig. 2b). Nuclear FT staining in epithelial cells was also noted. FT staining was also present in tissue stromal fibers of some IDC cases.

In agreement with these results, suggesting an effective iron 'reservoir' in lymphocytes and macrophages, hemosiderin detection through Perls staining demonstrated that a significantly higher proportion of carcinoma cases, when compared with control normal samples, present hemosiderin deposits in stromal inflammatory ($p = 0.002$) and epithelial cells ($p = 0.033$) (Fig. 5).

Comparative expression of iron-related proteins in pure DCIS lesions and DCIS in IDC

We demonstrated that the deregulated expression of iron-related proteins in breast cancer is not restricted to the tumor cells, but extends to the lymphocytes and macrophages in the tumor microenvironment. Given the fact that FPN1 expression in macrophages is particularly

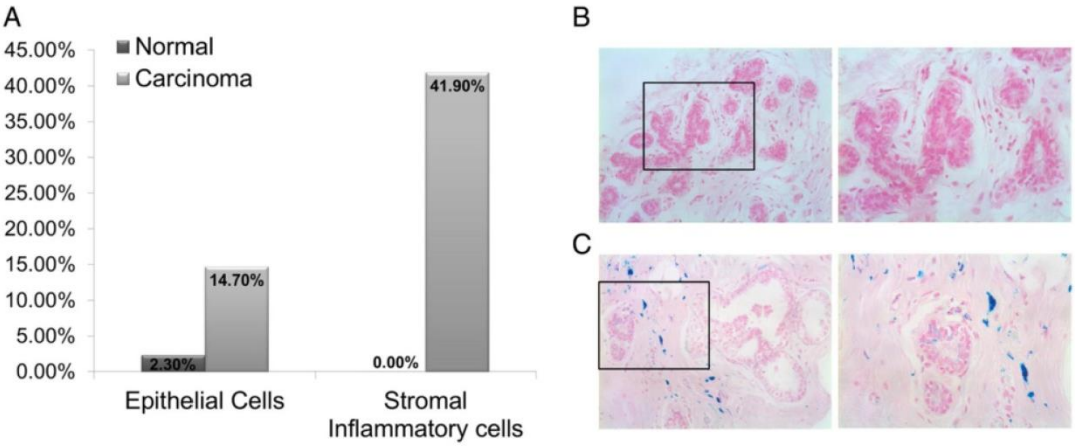

Fig. 5 Breast cancer tissue presents a higher accumulation of iron than normal breast. **a** Percentage of normal and breast cancer samples presenting hemosiderin deposits in epithelial and stromal inflammatory cells; **b-c** Representative images of Perls' iron staining of a normal (**b**) and DCIS (**c**) sample, showing pronounced deposition of hemosiderin in stromal inflammatory cells (arrows) and to a lesser extent in ductal epithelial cells (asterisk) (Original magnification × 200, ×400)

high in pre-invasive stages (DCIS), we sought to verify if these iron-related phenotypes were specific of pure DCIS or if they could also be observed in DCIS lesions adjacent to invasive ductal carcinomas (DCIS in IDC). The results are illustrated in Fig. 6.

Epithelial cells from DCIS pure lesions or from DCIS in IDC did not exhibit significant differences regarding the expression of the previously assessed iron-related proteins (Fig. 6a). Major differences were found, however, for tumor-associated lymphocytes and macrophages. Lymphocytes had a significantly higher expression of hepcidin ($p = 0.030$) and TFR1 ($p = 0.011$) in DCIS in IDC (Fig. 6c), while macrophages from DCIS pure lesions exhibited a higher expression of FPN1 than DCIS in IDC ($p = 0.036$) (Fig. 6b).

Imaging flow cytometry

In order to confirm the expression of FPN1 in epithelial cells, lymphocytes and macrophages from breast carcinoma samples and further explore its cellular distribution we resourced to imaging flow cytometry to relatively quantify it and determine its localization. For that purpose, we used OCT-frozen tissue from 6 patients with invasive ductal carcinomas, and a panel of antibodies to identify epithelial cells, T and B lymphocytes and macrophages (described in Methods). Furthermore, a mask to identify specifically the cell membrane and cytoplasm was built in IDEAS v6.0.348 software to evaluate FPN1 expression in each cell compartment. The ratio between the median FPN1 intensity in the cytoplasm and membrane was calculated as a putative surrogate for the iron

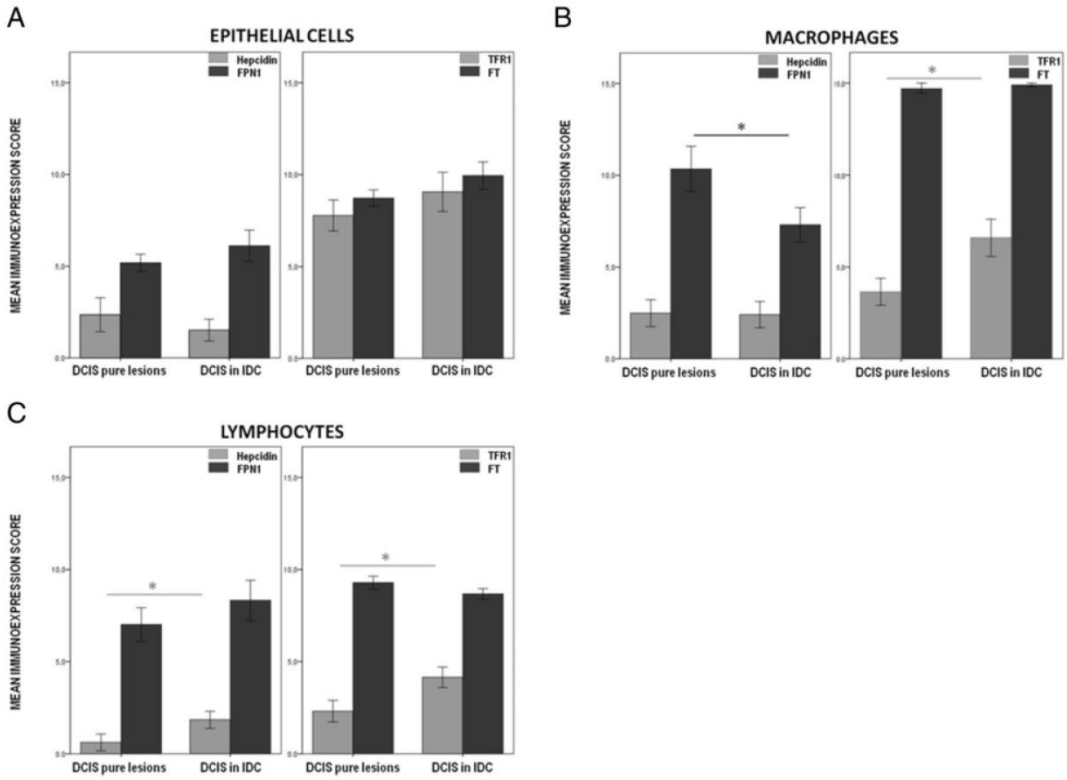

Fig. 6 Hepcidin/FPN1 and TFR1/FT phenotype dyads in ductal carcinoma *in situ* lesions (DCIS). A semi-quantitative method of assessing the immunoexpression in the TMA sections was applied by multiplying the area and intensity staining scores, as described in materials and methods. The scores ranged from 0 to 15. Graphs show Mean ± SEM. Significant differences are shown for comparison with the precedent group $*p < 0.05$, $**p < 0.01$, $***p < 0.001$, Mann Whitney's U test; **a** Immunoexpression score for breast epithelial cells in DCIS pure lesions and DCIS lesions in IDC for Hepcidin ($n = 35$), FPN1 (Ferroportin 1, $n = 35$), TFR1 (Transferrin Receptor 1, $n = 32$) and FT (Ferritin, $n = 36$); **b** Immunoexpression score for macrophages in DCIS pure lesions and DCIS lesions in IDC for Hepcidin ($n = 28$), FPN1 (Ferroportin 1, $n = 27$), TFR1 (Transferrin Receptor 1, $n = 30$) and FT (Ferritin, $n = 33$); **c** Immunoexpression score for lymphocytes in DCIS pure lesions and DCIS lesions in IDC for Hepcidin ($n = 26$), FPN1 (Ferroportin 1, $n = 31$), TFR1 (Transferrin Receptor 1, $n = 30$) and FT (Ferritin, $n = 33$)

export capacity of the cell. Representative images are shown in Fig. 7 and the results are summarized in Table 2. FPN1 expression could be detected by Imaging Flow Cytometry in epithelial cells, T lymphocytes, B lymphocytes and macrophages. Macrophages in breast cancer tissue presented the highest median fluorescence intensity of the cell types considered, as a confirmation of the results presented in Fig. 2b. The ratio between the median FPN1 intensity in the cytoplasm and membrane allowed us to notice that FPN1 expression was higher in the cytoplasm of all the cell types considered, when compared with the membrane.

Lymph nodes

Considering that the expression of iron-related proteins in lymphocytes and macrophages varied in different tumor microenvironments, we extended the observation to metastized and non-metastized lymph nodes from the original cohort of patients, whose primary tumors had been previously analyzed. Hepcidin, FPN1, TFR1 and FT immunoexpression were assessed in 14 non-metastized and 12 metastized lymph-nodes (Fig. 8) and semi-quantitatively scored (Table 3).

Hepcidin immunoexpression in lymph nodes was mostly restricted to macrophages and scarcely observed in lymphocytes. Metastized lymph nodes, however, presented a significantly higher immunoexpression of hepcidin in lymphocytes (B cell areas: $p = 0.005$; T cell areas: $p = 0.018$), than in non-metastized lymph-nodes.

FPN1 was uniformly expressed in all the cell types. In lymph nodes with sinus histiocytosis, lymphocytes and macrophages had a tendency to lower FPN1 expression. Remarkably, lymphocytes in metastized lymph nodes expressed 1.80-fold more FPN1 than in non-metastized ones, particularly in areas adjacent to the metastasis ($p = 0.002$). Expression assessment at the mRNA level confirmed a 1.48-fold increase in FPN1 expression in leukocyte areas of metastized lymph nodes, comparing with non-metastized lymph nodes ($p = 0.057$).

TFR1 was mostly expressed in macrophages and germinal center cells, particularly in non-metastized lymph nodes ($p = 0.026$). TFR1 was seldom expressed in T-cell areas, with no significant differences observed between metastized and non-metastized lymph nodes.

While the immunoexpression pattern of FT was similar to FPN1, a significantly higher expression of FT was observed for lymphocytes ($p < 0.001$), noted particularly near metastasis areas.

Clinicopathological data

The expression of iron-related proteins was finally correlated with clinicobiological markers of breast cancer behavior, specifically hormone receptor and HER2 status. Results of mean FPN1 expression values in epithelial cells and macrophages in DCIS and IDC lesions are shown in Table 4 in relation to the ER, PR and HER2 status. FPN1 expression in IDC lesions was significantly higher in ER negative ($p = 0.018$) and in HER2 positive cases ($p = 0.001$) in epithelial cells, whereas in DCIS lesions FPN1 expression was only associated with negative ER status in macrophages ($p = 0.033$). No associations were found between FPN1 expression and PR status. Regarding TFR1 expression, a significantly higher expression was seen in macrophages of negative PR DCIS cases ($n = 15$; $p = 0.039$) and in lymphocytes and macrophages of HER2 positive IDC cases (Ly: $n = 79$; $p = 0.028$; M0: $n = 79$; $p = 0.003$). A higher expression of FT in lymphocytes was observed in negative PR DCIS cases ($n = 15$; $p = 0.029$). All other comparisons were not statistically significant.

We next analyzed the expression of these iron-related proteins in relation to local and metastatic tumor growth in invasive tumors (Table 5). Tumor size was positively correlated with TFR1 expression in all the cell types considered (EC: $p = 0.027$; $r = 0.226$; Ly: $p = 0.041$; $r = 0.235$; M0: $p = 0.017$; $r = 0.274$).

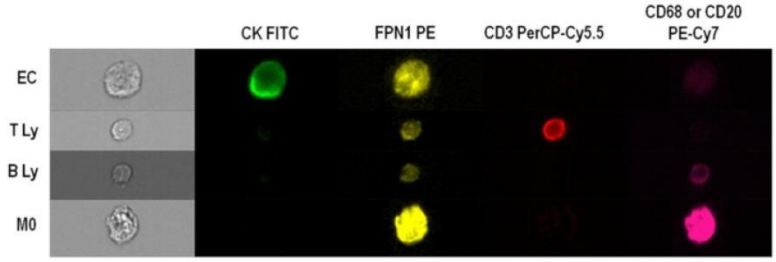

Fig. 7 Representative images of the FPN1 analysis by Imaging Flow Cytometry in breast cancer core biopsies. Epithelial cells (EC) are stained by an anti-cytokeratin (CK) FITC. T lymphocytes (T Ly) were identifiable by CD3 PerCP-Cy5.5, B lymphocytes (B Ly) by CD20 PE-Cy7 and macrophages (M0) by CD68 PE-Cy7 (Original magnification × 400)

Table 2 FPN1 median expression in IDC samples assessed by Imaging Flow Cytometry

Cell type	Mean number of cells on focus ± SEM	Total cell FPN1 PE MFI ± SEM	Cytoplasm FPN1 PE MFI ± SEM	Membrane FPN1 PE MFI ± SEM	Ratio cyt/memb FPN1 PE MFI ± SEM
EC	3935 ± 605	32.23 ± 4.26	46.08 ± 5.98	18.77 ± 2.32	2.47 ± 0.22
T Ly	53 ± 13	11.99 ± 0.63	15.7 ± 1.14	7.22 ± 0.22	2.31 ± 0.20
B LY	11 ± 7	15.09 ± 2.63	18.76 ± 2.04	9.41 ± 0.87	2.03 ± 0.03
M0	154 ± 38	69.82 ± 9.26	105.55 ± 23.82	53.11 ± 7.53	2.00 ± 0.001

Abbreviations: *FPN1* Ferroportin 1, *IDC* Invasive Ductal Carcinoma, *MFI* Median Fluorescence Intensity, *SEM* Standard Error of the mean, *Cyt* Cytoplasm, *Memb* Membrane, *EC* Epithelial Cells, *Ly* Lymphocytes, *M0* Macrophages

Lymph node involvement was not associated with the expression of these iron-related proteins in the primary tumor tissue. Of notice, the peripheral blood leukocyte count at the time of diagnosis was also correlated with the expression of TFR1 and FPN1 in primary tumor's lymphocytes (TFR1: $p = 0.001$; $r = 0.355$; FPN1: $p = 0.017$; $r = 0.274$) and macrophages (TFR1: $p = 0.002$; $r = 0.367$; FPN1: $p = 0.034$; $r = 0.244$).

Discussion

We would like to start this discussion by placing the present results in the growing interest on the tissue microenvironment contribution for malignancy [16, 29, 30]. Thus far, most of this interest has focused on cytokines and immune response to putative tumor antigens [31, 32]. Recently, however, interest has grown in the interaction of migrating cells to the tissue microenvironment,

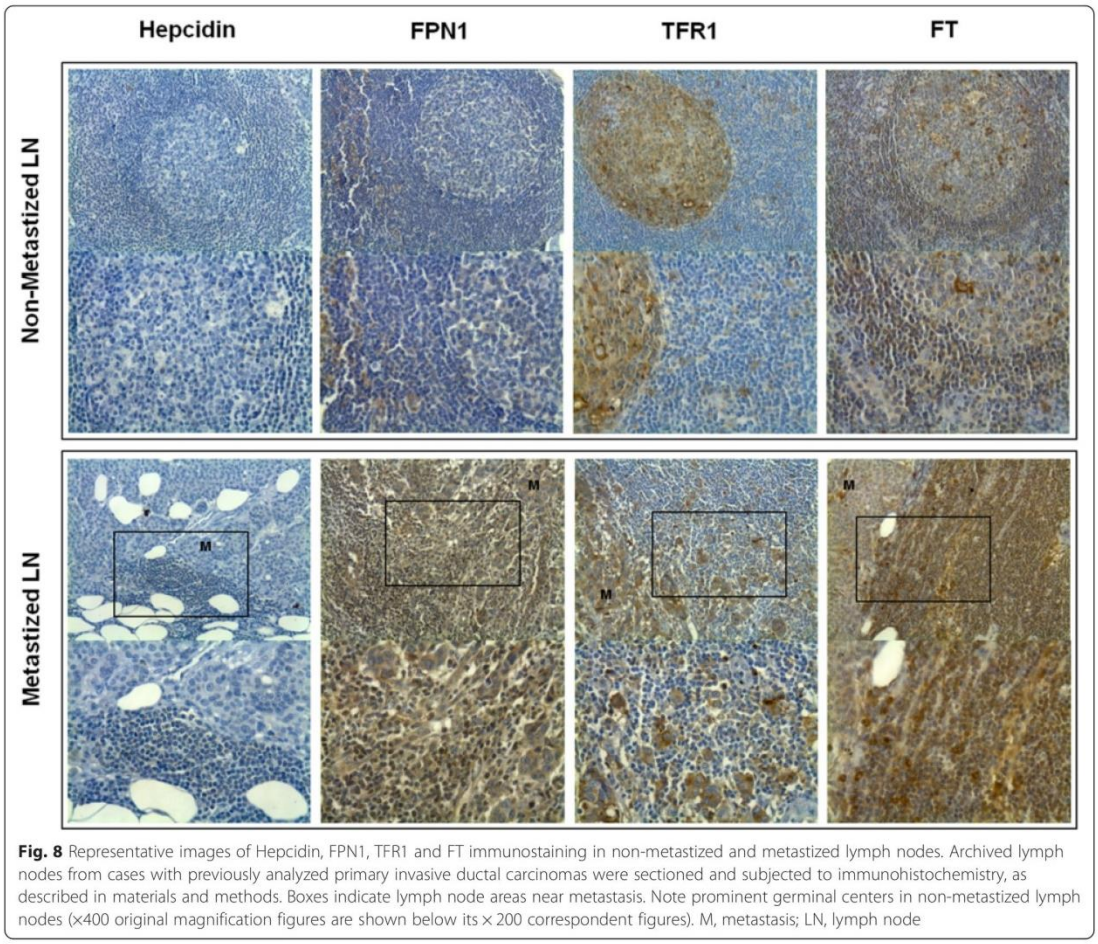

Fig. 8 Representative images of Hepcidin, FPN1, TFR1 and FT immunostaining in non-metastized and metastized lymph nodes. Archived lymph nodes from cases with previously analyzed primary invasive ductal carcinomas were sectioned and subjected to immunohistochemistry, as described in materials and methods. Boxes indicate lymph node areas near metastasis. Note prominent germinal centers in non-metastized lymph nodes (×400 original magnification figures are shown below its × 200 correspondent figures). M, metastasis; LN, lymph node

Table 3 Analysis of iron-related proteins expression in the LN of patients with IDC

		Non-metastized LN	Metastized LN	
	Cell type	Mean ± SEM	Mean ± SEM	*p*
Hepcidin	B cell areas	0.08 ± 0.08	0.62 ± 0.14	*0.005*
	T cell areas	0.15 ± 0.10	0.62 ± 0.14	*0.018*
	M0	2.00 ± 0.00	1.92 ± 0.08	ns
FPN1	B cell areas	1.38 ± 0.14	2.23 ± 0.17	*0.002*
	T cell areas	1.38 ± 0.14	2.23 ± 0.17	*0.002*
	M0	1.92 ± 0.08	2.23 ± 1.17	ns
TFR1	B cell areas	1.92 ± 1.18	1.08 ± 0.27	*0.026*
	T cell areas	1.00 ± 0.11	1.08 ± 0.08	ns
	M0	2.00 ± 0.11	2.31 ± 0.13	ns
FT	B cell areas	1.77 ± 0.12	2.77 ± 0.12	*<0.001*
	T cell areas	1.54 ± 0.14	2.62 ± 0.14	*<0.001*
	M0	3.00 ± 0.00	3.00 ± 0.00	ns

Abbreviations: *LN* Lymph Node, *IDC* Invasive Ductal Carcinoma, *SEM* Standard Error of the Mean, *M0* Macrophages, *FPN1* Ferroportin 1, *TFR1* Transferrin Receptor 1, *FT* Ferritin, *NS* Not Statistically Significant

namely macrophages, neutrophils and certain lymphocyte subsets [14, 33, 34].

Cells migrating to a tumor microenvironment must benefit in general from the tumor associated development of new vessels [35]. Angiogenesis is thought to provide nutritional advantage to the transformed malignant cell. Yet, very few studies have focused on an obvious nutrient associated with cell division, such as iron. The present study sought, to a certain extent compensate for that scanty interest.

Thus, we approached the question of the iron homeostasis deregulation in breast cancer by analyzing the specific iron-related phenotypes of different cell types present in the tumor tissue namely epithelial cells, lymphocytes and macrophages, and correlating the iron-related phenotypes with clinicopathological markers of disease prognosis. The analysis of iron-related phenotypes in breast ductal carcinoma epithelial cells confirmed previous observations that they display a phenotype of relative iron deficiency, characterized by a marked increase in TFR1 expression (for review see [36]) and downregulation of FT [21]. Although we observed an increase in hepcidin expression in breast cancer tissue, as previously described, we were not able to demonstrate a concomitant decrease in FPN1 expression in breast cancer epithelial cells, compared with normal epithelial cells [12, 13, 37]. Although not analyzed in our study, results from Wang et al. suggest that the 'iron-deficient' phenotype of breast cancer cells may be driven by the increased expression of the iron-regulatory protein (IRP) 2 [38]. The discrepancy found with previous reports regarding FPN1 might be due to the inclusion, in those studies, of different breast cancer types besides ductal carcinomas or by the assessment of FPN1 at the transcriptional level instead of the protein level, or still due to our limited number of samples. Also, we cannot exclude the influence of other regulatory mechanisms on FPN1 expression, other than hepcidin-mediated ferroportin 1 downregulation at the post-translational level, such as epigenetic mechanisms, deregulation of Nrf2 and MZF-1 expression [37] or a HIF-2α dependent pathway [39].

The analysis of iron-related phenotypes in stromal inflammatory cells revealed that infiltrating macrophages and lymphocytes display an "iron-donor" phenotype with increased expression of both FPN1 and FT concomitant with an activation profile reflected by a higher expression of hepcidin and TFR1. The increased FPN1 expression was particularly evident in macrophages of DCIS lesions (see Figs. 2 and 6), but was also clear in

Table 4 FPN1 expression in epithelial cells and macrophages in carcinomas according to clinicopathological variables

	DCIS (Mean ± SEM)				IDC (Mean ± SEM)			
Clinicopathological variable	EC	*Significance level.*	M0	*Significance level.*	EC	*Significance level.*	M0	*Significance level.*
ER status	*n* = 22	*ns*	*n* = 14	*p = 0.033*	*n* = 103	*p = 0.018*	*n* = 73	*ns*
ER-	6.29 ± 0.65		13.04 ± 0.87		7.47 ± 0.88		7.17 ± 0.95	
ER+	4.51 ± 0.43		7.75 ± 2.14		5.18 ± 0.31		6.01 ± 0.34	
PR status	*n* = 22	*ns*	*n* = 14	*ns*	*n* = 103	*ns*	*n* = 73	*ns*
PR-	6.01 ± 0.61		11.59 ± 1.64		6.47 ± 0.65		7.25 ± 0.77	
PR+	4.48 ± 0.46		9.30 ± 1.80		5.40 ± 0.36		5.83 ± 0.35	
HER2 status	*n* = 22	*ns*	*n* = 14	*ns*	*n* = 101	*p = 0.001*	*n* = 73	*ns*
HER2-	5.76 ± 0.63		9.00 ± 2.45		5.21 ± 0.35		6.02 ± 0.38	
HER2+	4.98 ± 0.57		11.76 ± 1.34		7.47 ± 0.64		6.89 ± 0.74	

Abbreviations: *DCIS* Ductal Carcinoma *In Situ*, *IDC* Invasive Ductal Carcinoma, *ER* Estrogen Receptor, *PR* Progesterone Receptor, *HER2* Human Epidermal growth factor Receptor 2, *EC* Epithelial Cells, *Ly* Lymphocytes, *M0* Macrophages, *FPN1* Ferroportin 1, *TFR1* Transferrin Receptor 1, *FT* Ferritin, *ns* Not Statistically Significant, *SEM* Standard Error of the Mean

Table 5 Correlation table between tumor size and TFR1 expression

	TFR1 (Mean ± SEM)					
Clinicopathological variable	EC	Corr. Coeff. Sig	Ly	Corr. Coeff. Sig	M0	Corr. Coeff. Sig
Tumor size	*n* = 96	*p* = 0.027	*n* = 76	*p* = 0.041	*n* = 76	*p* = 0.017
T1	6.69 ± 0.56		3.37 ± 0.31		5.39 ± 0.47	
T2	7.83 ± 0.93		4.99 ± 0.57		6.94 ± 0.63	
> T3	9.00 ± 4.83		3.47 ± 0.65		7.00 ± 1.34	

Abbreviations: *EC* Epithelial Cells, *Ly* Lymphocytes, *M0* Macrophages, *TFR1* Transferrin Receptor 1, *SEM* Standard Error of the Mean

lymphocytes, not only in the primary tumor site but also in metastized lymph nodes (see Figs. 2c and 8 and Table 3). The simultaneous overexpression of TFR1 and FT, and hepcidin and FPN1 in lymphocytes and macrophages questions the established principles of iron-related proteins' regulation at the post-transcriptional and –translational levels. Considering the role of IRPs 1 and 2 in a situation of high iron levels, as expected in a breast cancer setting, IRP binding to 5′- untranslated region (UTR) of FT and lack of stabilization at the 3′-UTR of TFR1 would lead to an increased FT translation, with a concomitant decrease in TFR1. Unexpectedly, this was not observed, Although TFR1 is classically viewed, in this context, for its role in iron acquisition and malignant cell nutrition, there is evidence showing an alternative role for TFR1 in the activation of T cells, independently of iron-uptake [36, 40, 41]. Furthermore, we cannot disregard the fact that FT detection was achieved with a polyclonal antibody, not discriminating the heavy and light subunits, which could be argued as not reflecting iron accumulation in these cells. However, the fact that we also demonstrated that over 40 % of DCIS and IDC samples present stromal inflammatory cells with hemosiderin deposits supports our hypothesis that these cells may constitute an effective iron reservoir potentially contributing to tumor nutrition. The observed concurrent increased expression of hepcidin and FPN1 in lymphocytes and macrophages of breast ductal carcinomas may also argue in favor of such a nutritional role. Studies from others have also demonstrated a similar hepcidin-independent mechanism of iron export, reflecting a role for heme. They showed that heme derived from erythrophagocytosis can stimulate FPN1 transcription in primary cultures of bone marrow derived macrophages and that hepcidin was not able to block iron-heme export during erythrocyte-iron recycling by macrophages [42, 43].

Markers of iron deregulation in stromal cells were also found here significantly associated with other clinicopathological markers of poor prognosis, namely hormone receptor status negativity and tumor size (Tables 4 and 5). Several studies had already focused on the establishment of associations between the immune profile of the infiltrating leukocytes in the tumor and established clinicopathological variables of breast cancer outcome [44–46] but very few have approached the association with iron-related proteins. Interestingly, Britten et al. had already demonstrated that spleens from patients with Hodgink's disease presented a higher expression of ferritin in macrophages, particularly around tumor nodules [47]. The present results point to the fact that deregulation of iron metabolism occurs not only at the primary tumor microenvironment, but also in preferential metastatic niches. To our knowledge, only Jezequel and coworkers have previously described the expression of an iron-related protein (ferritin light-chain, FTL) in stromal cells as a prognostic marker in node-negative breast cancer patients [22]. In the present study, and for the first time, an association between the expression of iron-related proteins in lymphocytes and macrophages and negative hormone receptor status in DCIS and tumor size was demonstrated. Moreover, neither the expression of FPN1 and FT in axillary lymph nodes nor the association of FPN1 and FT overexpression in lymphocytes with the presence of breast cancer metastasis has been previously described. In summary, these significant associations observed for lymphocytes and macrophages reinforce a reason for interest in their contribution for the tumor microenvironment.

This study raises some new questions that deserve to be analyzed. The first one is how stromal cells acquire their "iron donor" phenotype. Are they responding locally to signals derived from cancer cells? Are they mobilized from the peripheral blood with this phenotype? Further studies should be performed to clarify this question. One may consider however that, at the systemic level, macrophages and lymphocytes constitute important iron storage compartments involved, respectively, in the recycling of iron from senescent erythrocytes and in the uptake of non-transferrin-bound iron [48–50]. Based on the original hypothesis of de Sousa of the immune system in the surveillance of the potential iron toxicity associated with red blood cell circulation and more recently extended by Pinto et al. work in 2014 [49, 51], this circulating compartment might, indeed, be responsible for delivering iron locally in situations of increased blood flow, such as in tumor-derived angiogenesis, and hence contribute to tumor sustained growth through iron

nutrition [52]. This notion is further supported by our present data showing a highly significant correlation between the number of circulating leukocytes at the time of diagnosis and the high expression of FPN1 and TFR1 in breast cancer infiltrating lymphocytes and macrophages.

Conclusion

In summary, the results presented here confirm that the deregulation of iron metabolism is an aspect common to several cellular types of breast cancer tissue, and not restricted to epithelial cells. Moreover, this deregulation of iron-related proteins in infiltrating lymphocytes and macrophages add evidence to the view that stromal cell responses in the breast microenvironment may contribute critically to tumor progression [53, 54].

With the present cross-sectional approach it was not possible to establish a real timeline for breast cancer development and progression. Moreover, the fact that this is an anonymous cross-sectional study without access to follow-up data, has limited the comprehension of the value of iron-related alterations in infiltrating lymphocytes and macrophages of the breast tumor microenvironment. Subsequent prospective studies monitoring the expression of these iron-related proteins in patients are still needed to validate the significance of local iron-profiles as relevant markers of breast disease progression. Furthermore, the analysis of IRP expression in specific breast tissue cell types may provide additional insight into the regulation of iron homeostasis in breast cancer. Future in vitro studies should be designed in order to confirm not only the capacity of lymphocytes and macrophages to donate iron to breast epithelial cells but also to explore how malignant cells could influence their environment in order to acquire iron beyond stimulating angiogenesis.

Ethics approval

This project was approved by the following ethical boards: Centro Hospitalar do Porto Research Ethics Health Committee (references 228-CES; 203-CES) and by Centro Hospitalar do Porto Department of Education, Development and Research (references 152-DEFI; 135-DEFI). Informed consent from the patients whose tissue was archived was not required as per Ethics Board guidelines. Patient informed consent for the use of breast core biopsies for flow cytometry studies was obtained.

Abbreviations

BSA: bovine serum albumin; DCIS: ductal carcinoma in situ; EC: epithelial cell; ER: estrogen receptor; FFPE: formalin-fixed paraffin-embedded; FPN1: ferroportin 1; FT: ferritin; H&E: hematoxylin and eosin; HER-2: human epidermal growth factor 2; IDC: invasive ductal carcinoma; Ly: lymphocyte; M0: macrophage; OCT: optimum cutting temperature; PR: progesterone receptor; TFR1: transferrin receptor 1; TMA: tissue microarray; UTR: unstranslated region; WBC: white blood cell.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

OM conceived, designed and performed the experiments, analyzed the data and wrote the manuscript. GP analyzed and interpreted the data and wrote the manuscript. AR and FF performed the histological techniques. ACP collected the biopsies and contributed with reagents. MGL performed the data acquisition in the Imaging Flow Cytometry experiment and analyzed the data. PS performed the Laser Capture Microdissection. BMS conceived experiments and contributed with reagents, materials and analysis tools. CL conceived and designed experiments, analyzed the slides and contributed with materials and analysis tools. All authors have read, revised and agreed to the publication of the manuscript.

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Chapter 4

CCL2 Expression in Breast Ductal Carcinomas: a Novel Modulator of Local Iron Homeostasis?

CCL2 EXPRESSION IN BREAST DUCTAL CARCINOMAS: A NOVEL MODULATOR OF LOCAL IRON HOMEOSTASIS?

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Keywords: Breast; iron; macrophages; CCL2; tumor microenvironment; ferroportin 1

Abstract

Tumor microenvironment is pivotal for neoplastic progression. Recent studies reported that stromal inflammatory cells may supply iron to tumors. Recruitment of inflammatory leukocytes into the tumor milieu involves chemokines, such as CCL2. Our aim was to understand whether CCL2 expression, in the breast cancer model, could be related to tissue iron status.

A total of 21 normal samples, 27 ductal carcinomas *in situ* (DCIS) and 35 invasive ductal carcinomas (IDC) were used for tissue microarray construction. DAB-enhanced Perls' staining and immunohistochemistry for CCL2, ferroportin 1 (FPN1), CD68, CD4, CD8 and FoxP3 were performed. The common HFE variants (p.C282Y and p.H63D) were characterized by PCR-RFLP.

Comparing with normal samples, a higher proportion of carcinomas presented iron deposition. Epithelial CCL2 expression was associated with the infiltration of CCL2-positive macrophages, CD4-positive lymphocytes and total lymphocyte infiltration. The median epithelial CCL2 expression was higher in the presence of iron in stromal inflammatory cells and its expression was associated with FPN1 expression in lymphocytes. No associations were found between HFE variants and iron deposition or CCL2 expression. Moreover, epithelial CCL2 expression was significantly associated with the estrogen-receptor negative status.

Overall this study provides further evidences supporting the notion that tumor-infiltrating inflammatory cells display deregulated iron homeostasis, possibly favoring the supply of iron to breast tumor cells. Associations found between epithelial CCL2 expression and infiltration of stromal inflammatory cells suggest the existence of a paracrine signaling pathway where CCL2 may play an indirect role regulating tumor iron nutrition and progression.

Introduction

Iron is an essential microelement, vital for a variety of biological and cellular processes (1). However, iron is also toxic, due to its participation in the formation of mutagenic hydroxyl radicals (2), suppression of the host immune response (3) and by acting as a limiting nutrient for proliferating tumor cells (4). Epidemiological, experimental and clinical evidences support the hypothesis that iron is strongly associated with breast cancer initiation and progression (5-7). Results from several studies provide evidence that tumor cells behave as iron deficient and are able to undermine the tightly physiological process of iron regulation, by increasing iron acquisition and retention, independently of its intracellular iron levels (1, 8). Upregulation of the transferrin receptor 1 (9), production of transferrin (10) and downregulation of ferroportin 1 (FPN1) (8, 11) have been reported in breast cancer as signs of deregulation of iron homeostasis.

Accumulating evidences are bringing some new insight on the role of tumor-associated inflammatory cells. A multitude of studies reported that malignant cells require an appropriate support structure to successfully acquire a full malignant potential (12, 13). Tumor-associated macrophages constitute up to 50% of the tumor mass and contribute to each stage of tumor progression, by promoting matrix remodeling, tumor cell invasion, neovascularization and metastasis (14). The M1/M2 dichotomy is frequently used to characterize these cells and polarization is dependent on specific stimuli (15). Macrophages are also fundamental cells for regulation of iron homeostasis (16) and polarization can have important effects on iron metabolism (17). Conversely, iron can directly influence macrophage polarization (17).

In a recent study performed by Marques and colleagues, iron-profiles of breast cancer tumor-associated cells were analyzed (18). Data obtained from this study suggest lymphocytes and macrophages exhibit an “iron-donor” phenotype, characterized by increased FPN1 and ferritin expression in carcinomas comparing to their normal counterparts, reinforcing the role of these immune cells as potential iron suppliers.

One of the chemokines known to polarize macrophages into an M2-like phenotype is CCL2 (19). CCL2 (chemokine [(C-C motif)] ligand 2) is known to play a role in leukocyte recruitment into the tumor microenvironment and is one of the most studied chemotactic cytokines in the context of tumor progression, particularly in breast cancer (20). The modulation of CCL2 expression by cellular iron status was already demonstrated by Mitchell and colleagues, in a study using neuroblastoma and astrocytoma cells. Regardless of the malignant cell type and HFE genotype, CCL2 secretion was influenced by iron status, with the exception of cells expressing the p.C282Y HFE variant. In the non-

malignant cell line also included in this study, this HFE variant did not appear to alter the impact of cellular iron status on CCL2 expression (21). In other studies, not related to neoplastic disease, iron supplementation or chelation also influenced CCL2 protein levels (22-25).

Given the recent evidences suggesting that tumor-associated macrophages and lymphocytes are involved in iron deregulation, and the key role of CCL2 as a macrophage chemoattractant, we investigated whether CCL2 expression is associated with tissue iron status in breast cancer. We analyzed CCL2 expression, assessed the local iron deposition and the expression of FPN1 in stromal inflammatory cells and looked for correlations between CCL2 expression and iron profiles in epithelial and stromal inflammatory cells, especially with proteins known to be deregulated during neoplastic progression. In addition, we analyzed whether CCL2 expression could be associated with the presence of the HFE allele variants and with clinical-pathological parameters.

Materials and Methods

Breast Tissue samples

This study was approved by the Ethics Committee of the Porto Hospital Centre, Portugal. A total of 83 samples, consisting of 35 invasive ductal carcinomas (IDC), 27 ductal carcinomas *in situ* (DCIS) and 21 aesthetic reduction mammoplasty samples were selected. Corresponding formalin-fixed, paraffin-embedded (FFPE) tissue blocks and hematoxylin-eosin (H&E) slides were collected from the archives of the same Pathology Service (Porto). Selected tumor samples were representative of primary breast tumors, collected from women diagnosed between 2004 and 2009, not previously subjected to neoadjuvant treatments.

Clinical information

Clinical and pathological parameters were retrieved from interin pathological reports. These included histological diagnosis, tumor size, lymph node involvement, estrogen (ER) and progesterone receptors (PR) and human epidermal growth factor receptor 2 status (HER-2). Peripheral white blood cell (WBC) count was also available.

Tissue microarray construction

H&E slides corresponding to the original FFPE blocks were analyzed by a pathologist, in order to select normal, *in situ* and invasive areas. The selected area was removed from the donor block and placed into the recipient block, following a previously designed map. Normal liver and lymph node tissues were also inserted, as internal

controls for immunostainings. A total of 452 cores were used for tissue microarray construction, out of which 297 were available for further pathological analysis. TMA blocks were cut in 2 µm sections and H&E staining was performed. These slides were used for the pathologist to classify the cores, without knowledge of the type of donor sample. A designation of “Normal in DCIS” or “Normal in IDC” was attributed to the cores with non-neoplastic tissue, retrieved from DCIS samples or IDC samples, respectively. Moreover, designation of “pure DCIS” or “pure IDC” was attributed to cores representative of the original sample. A total of 21 normal, 17 normal in DCIS, 27 pure DCIS, 28 normal in IDC and 35 pure IDC samples were analyzed.

DAB-enhanced Perls’ Prussian Blue staining

In order to assess hemosiderin deposition, DAB-enhanced Perls’ Prussian Blue was performed, as described in (26). TMA blocks were cut in 4 µm sections and mounted in adhesive slides coated with 3-Aminopropyltriethoxysilane (APES) (A3648, Sigma-Aldrich, St. Louis, MO, USA). Slides were stained with 1% potassium cyanide in distilled water, for 40 minutes, and washed in distilled water. Samples were treated with methanol containing 0.01M NaN_3 and 0.3% H_2O_2 for 75 minutes and washed with PBS. For the intensification reaction, slides were incubated with a solution containing 0.025% 3.3' – Diaminobenzidine-tetrahydrochloride (3.3’-DAB-4HCl) and 0.005% H_2O_2 in PBS, for 40 minutes. The reaction was stopped by rinsing in distilled water and tissues were counterstained with nuclear red (Merck Millipore, Darmstadt, Germany). A negative control was used by omitting the incubation with potassium ferrocyanide. Degradation of H_2O_2 by potassium ferrocyanide, together with the addition of DAB, leads to the formation of a dark-brown coloration due to the polymerization of the chromogen (27).

Immunohistochemistry

CCL2, CD68 (macrophages), CD4 (T-helper lymphocytes), CD8 (cytotoxic T lymphocytes) and FoxP3 (regulatory T lymphocytes) immunohistochemistry was performed on TMA sections. Mouse monoclonal anti-human CCL2 (ab9858, 1:25, Abcam, Cambridge, United Kingdom), mouse monoclonal anti-human CD68 (clone KP-1, 1:2000, Cell Marque Corporation, California, USA,), polyclonal anti-human CD4 (clone H-370; 1:250; Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA), monoclonal anti-human CD8 (clone C8/144B; 1:100; Cell Marque, Rocklin, California) and monoclonal anti-human FoxP3 (clone 236A/E7; 1:200; eBioscience, San Diego, USA) antibodies were used. FPN1 immunohistochemistry was performed as previously described (18). Antigen retrieval was performed using Dako Target Retrieval Solution (Agilent Technologies, Denmark), at 10% in distilled water, in a water bath at 100ºC, for 25 minutes.

Immunostaining was performed using the Novocastra Novolink Detection System (Leica Systems). All antibodies were used in single immunohistochemistry procedures, except for CD4 and FoxP3, which were simultaneously incubated in the same slide. Revelation was performed using DAB and counterstained with Mayer's hemalum solution (Merck Millipore, Billerica, MA, USA). Antibody dilutions were determined in tissue positive controls and negative controls were included by replacing the primary antibody with an antibody of the same immunoglobulin isotype.

Immunostaining analysis

For the evaluation of CCL2 and FPN1 staining in epithelial and stromal inflammatory cells, a semi-quantitative method was used. The following cut-off values were established for the intensity: score 0 = no staining; 1 = weak; 2 = moderate and 3 = strong staining. For the area stained the following cut-off values were considered: score 0 = 0%; 1 = 1-10%; 2 = 11-20%; 3 = 21-35%; 4 = 36-50% and 5 = >50%. The final score was obtained by multiplying the area and the intensity scores. CCL2+ and CD68+ macrophages, plus CD4+, CD8+ and CD4+FoxP3+ T-lymphocytes were counted in 5 high power fields (HPF), which are representative 400× fields in each spot. The values obtained for each antibody, from the 5 HPFs, were added in order to obtain the total cell count. Cores from the same donor tissue diagnosed with the same histological type were grouped and their mean score calculated.

Genotyping

Genomic DNA was extracted from FFPE sections using the Ultraprep Tissue DNA kit (AHN Biotechnologie, Nordhausen, Germany). PCR was carried out in 15.5μL reaction volumes, containing 2μL of the genomic DNA template, 7.5μL of MasterMix DNA polymerase, 1μL of Q-solution (both from Qiagen Multiplex PCR kit, Valencia, CA, USA) and 1μL of each of forward and reverse primers. For the detection of the C282Y polymorphism the following primers were used: 5'-CAAGTGCCTCCTTTGGTGAAGGTGACACAT-3' as the forward primer and 5'-CTCAGGCACTCCTCTCAACC-3' as the reverse primer (Metabion, Steinkirchen, Germany). These primers amplify a fragment with 343 bp and RsaI was the endonuclease used. For the HFE H63D polymorphism, the following forward and reverse primers' sequences were used: 5'-ACATGGTTAAGGCCTGTTGC-3' and 5'-GCCACATCTGGCTTAAAATT-3' (Metabion, Steinkirchen, Germany). These primers amplify a fragment with 294 bp and the endonuclease MboI was used for restriction. These primers were chosen according to the work of Feder *et al.* (28). A "hotstart" polymerase enzyme was used, so an initial step of 95ºC, for 15 minutes, was executed.

Following this, 36 cycles of denaturation at 94ºC for 30 seconds, annealing at 58ºC for 90 seconds and extension at 72ºC for 90 seconds were performed. Reaction was extended at the end for 10 minutes at 72ºC. The same PCR program was used for both HFE variants.

Statistical analysis

Data was analyzed with IBM SPSS Statistics 18.0 (SPSS Inc., IBM, Chicago, IL, USA). Graphically, results are presented showing median values and more or less 95% CI (confidence intervals). Pearson's Chi-Square was used to evaluate the differences between categorical variables. Sample distributions were compared using Kruskal-Wallis or Mann-Whitney tests. The first one was used to determine statistical significance when 2 data sets were compared and Kruskal-Wallis test to compare between 3 set of data. The Dunn-Bonferroni post hoc method was performed following a significant Kruskal-Wallis test to determine the statistical significant between 2 specific groups. The Spearman's rank correlation coefficient was used to evaluate the relationship between variables. Statistical significance was accepted at $p<0.05$.

Results

Tissue iron deposition demonstrated by DAB-enhanced Perls'

The presence of hemosiderin deposition was assessed in epithelial and in stromal inflammatory cells by the DAB-enhanced Perls' method. When present, hemosiderin deposits were found in the cytoplasm (Figure 1a and Figure 1b).

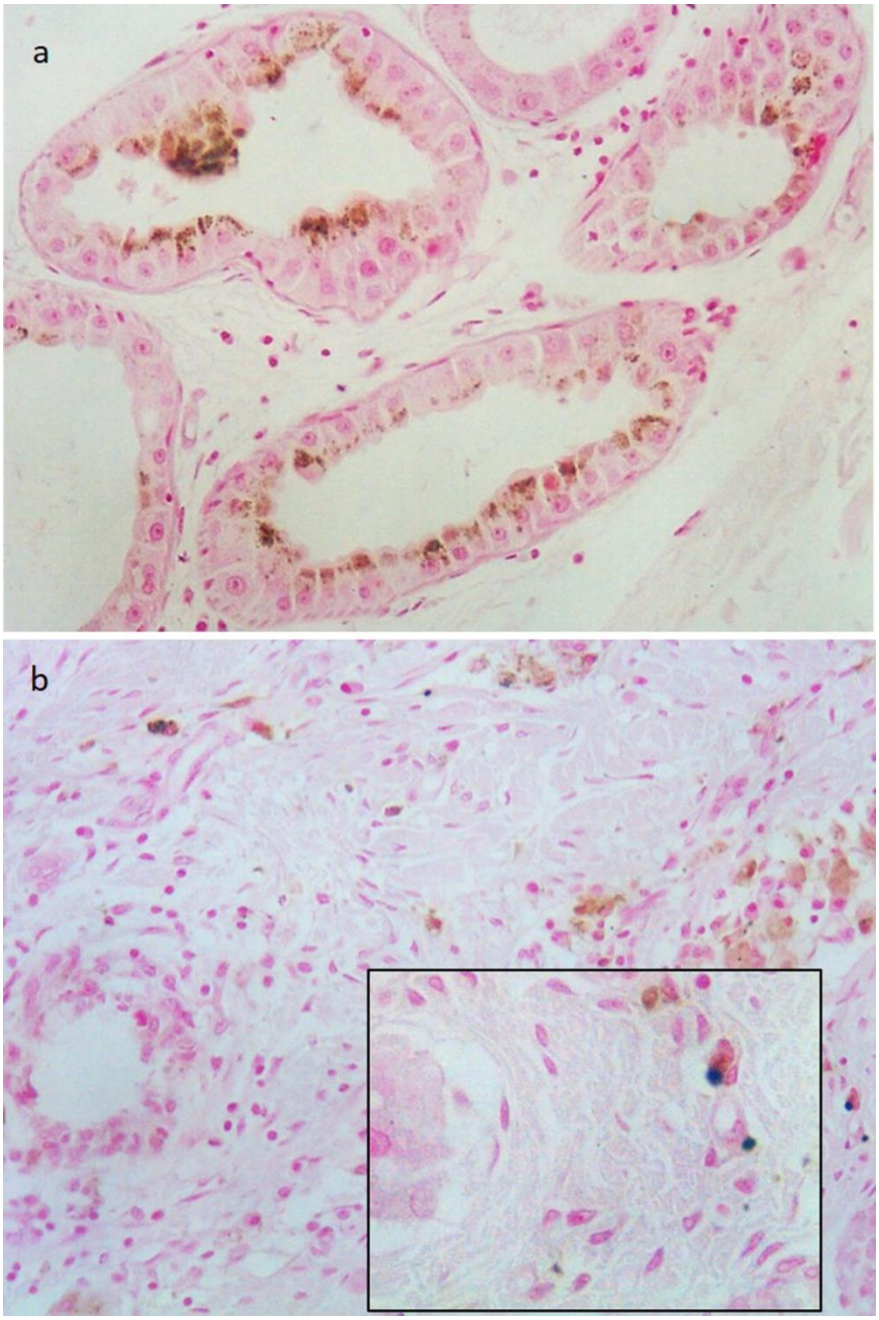

Figure 1. Evidence of hemosiderin deposition in a DCIS lesion (a) and in an IDC lesion (b). DAB-enhanced Perls' staining of breast tumors, particularly in epithelial (a) and stromal inflammatory cells (b). Original magnification of 200X (a and b) and 400X (inset).

Tissue iron deposition was evaluated by assessing the percentage of cases diagnosed within the same lesion presenting iron deposits in epithelial or stromal inflammatory cells. Regarding pure lesions, the presence of hemosiderin deposits in epithelial and stromal inflammatory cells was more evident in carcinomas than in normal tissue (Figure 2). Moreover, statistically significant differences were found for iron deposition in stromal inflammatory cells, between normal and pure DCIS ($p=0.011$) and

normal and pure IDC (p=0.001) (Figure 2). The presence of hemosiderin deposits in epithelial and in stromal inflammatory cells was more evident in non-neoplastic tissue adjacent to carcinomas than in normal tissue from reduction mammoplasty samples (p=0.037) (Figure 3). Differences in the percentage of samples presenting iron deposition in stromal inflammatory cells were statistically significant when comparing normal tissue to normal tissue adjacent to IDC lesions (p=0.011) (Figure 3).

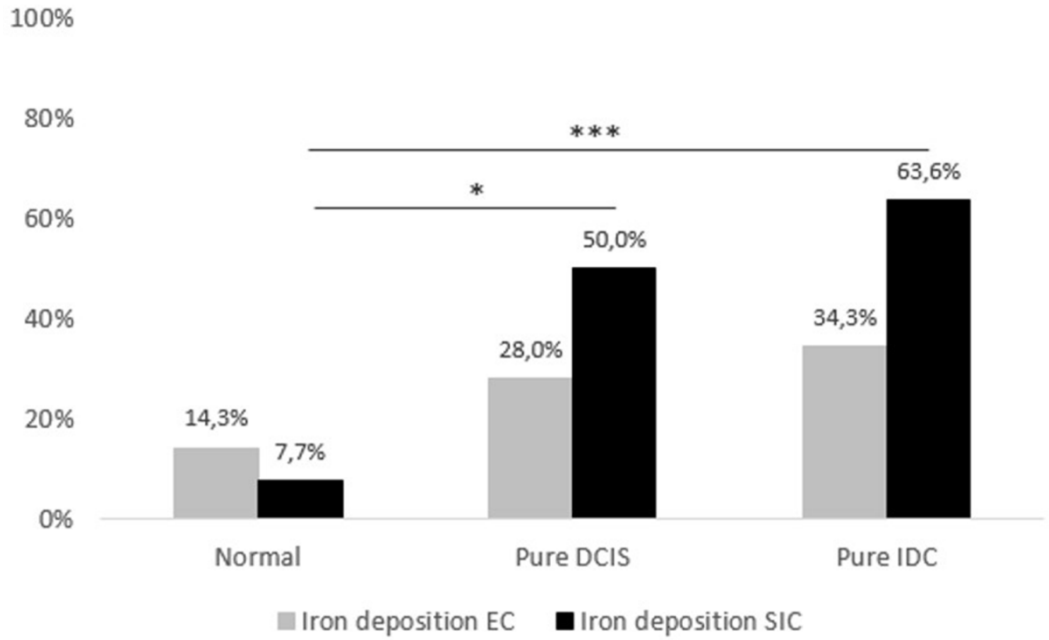

Figure 2. Presence of hemosiderin deposition in pure lesions of the breast, in epithelial (grey) and in stromal inflammatory cells (black). Chi-square test for iron deposition in stromal inflammatory cells in pure lesions: between normal and pure DCIS p=0.011; between normal and pure IDC p=0.001 (*p< 0.05, **p< 0.01, ***p< 0.001). Abbreviations: EC, epithelial cells; SIC, stromal inflammatory cells; DCIS, ductal carcinoma *in situ*; IDC, invasive ductal carcinoma.

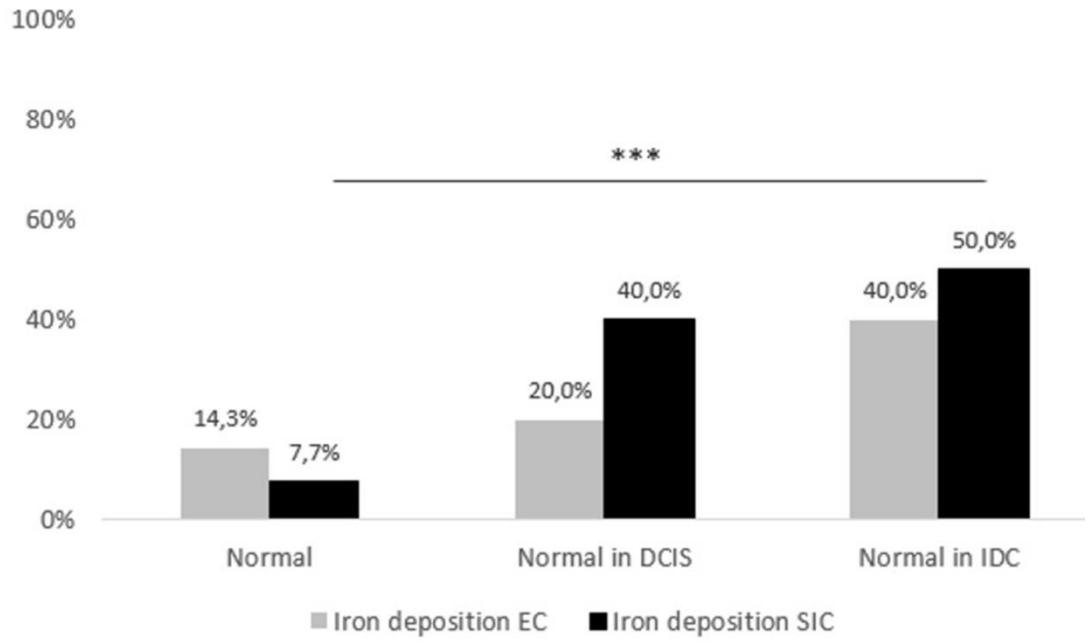

Figure 3. Presence of hemosiderin deposition in the tissue adjacent to breast lesions, in epithelial (grey) and in stromal inflammatory cells (black). Chi-square test for iron deposition in stromal inflammatory cells: p=0.011 (*p< 0.05, **p< 0.01, ***p< 0.001). Abbreviations: EC, epithelial cells; SIC, stromal inflammatory cells; DCIS, ductal carcinoma *in situ*; IDC, invasive ductal carcinoma.

Association of CCL2 expression in epithelial and stromal inflammatory cells with malignancy

CCL2 has been implicated in breast cancer progression, mainly because of its role as a leukocyte chemoattractant. Comparing with normal tissue, CCL2 immunostaining was higher in tumor samples. Expression of CCL2 in epithelial cells was positively correlated with malignancy (n=80; p=0.007; r= 0.299), as assessed by the Spearman's rank correlation test. Kruskal-Wallis test was performed and revealed differences in the epithelial CCL2 expression between sub-groups of pure lesions (p=0.022). Dunn-Bonferroni correction showed that statistical significance was only reached when comparing normal to pure IDC lesions (p=0.017) (Figure 4a). Increased expression of CCL2 was already evident in the non-neoplastic tissue adjacent to carcinomas, but we found no statistically significant differences in epithelial CCL2 expression when comparing normal tissue from reduction mammoplasties to non-neoplastic tissue adjacent to carcinomas (p=0.067)) (Figure 4b).

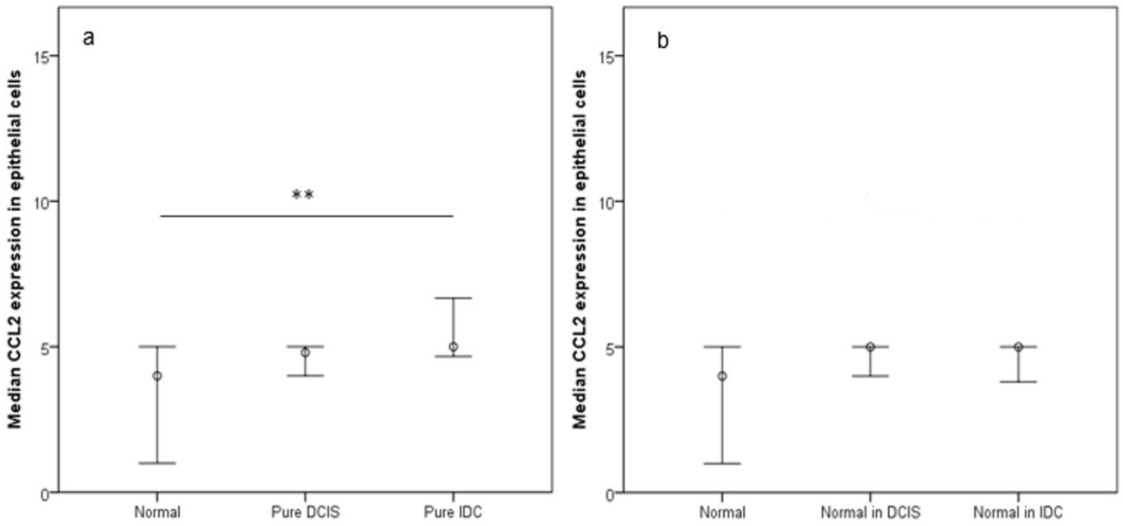

Figure 4. Median epithelial CCL2 expression in (a) pure lesions of the breast and (b) in non-neoplastic tissue adjacent to breast lesions. (a) Dunn-Bonferroni test: between normal and pure IDC lesions p=0.007. Error bars: 95% CI. (*p< 0.05, **p< 0.01, ***p< 0.001). Abbreviations: DCIS, ductal carcinoma in situ; IDC, invasive ductal carcinoma.

Local infiltration of CCL2-positive macrophages was also analyzed by immunohistochemistry. Total macrophage count presenting CCL2 immunostaining was strikingly higher in pure DCIS lesions and was also positively associated with increased malignancy (n=74; p=0.004; r= 0.335) (Figure 5a). Kruskal-Wallis test revealed statistically significant differences between sub-groups of pure lesions (p<0.001). The Dunn-Bonferroni test revealed that differences in normal samples comparing with pure DCIS and normal comparing with pure IDC samples were statistically significant (Figure 5a). Kruskal-Wallis test revealed no statistical differences in the infiltration of CCL2-positive macrophages between normal tissue from aesthetic reduction mammoplasties and non-neoplastic tissue adjacent to carcinomas (p=0.069) (Figure 5b).

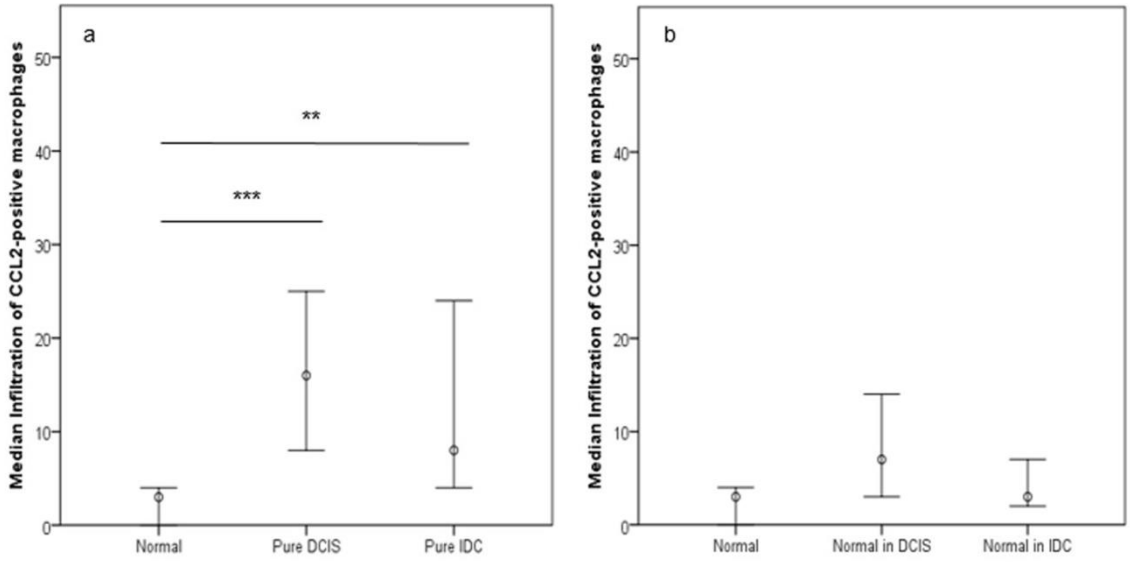

Figure 5. Infiltration of CCL2-positive macrophages in (a) pure lesions and (b) in non-neoplastic tissue adjacent to breast lesions. (a) Dunn-Bonferroni test: between normal and pure DCIS p<0.001; between normal and pure IDC p=0.001. Error bars: 95% CI. (*p< 0.05, **p< 0.01, ***p< 0.001, versus precedent group) Abbreviations: DCIS, ductal carcinoma in situ; IDC, invasive ductal carcinoma.

Association of total macrophage infiltration with malignancy

CD68 immunohistochemistry was performed to facilitate macrophage counting and assess total macrophage infiltration. Macrophage infiltration was more evident in carcinomas than in normal mastectomy samples. According to the Spearman's test, the number of macrophages was positively associated with malignancy (n=75; p<0.001; r=0.630). Kruskal-Wallis test revealed statistically significant differences between sub-groups of pure lesions (p<0.001). Dunn-Bonferroni test revealed significant differences when comparing normal to pure DCIS (p=0.003) and normal comparing to pure IDC (p<0.001). In the hypothetically normal tissue adjacent to carcinomas, the Kruskal-Wallis test revealed no statistically significant differences between sub-groups of lesions (p=0.099).

Association of CCL2 expression with macrophage and lymphocyte infiltration

Epithelial CCL2 expression was not correlated with total macrophage infiltration (n=75; p=0.609), but was associated with infiltration of CCL2+ macrophages (n=73; p=0.022). Infiltration of CCL2+ macrophages was positively associated with total macrophage infiltration (n=73; p<0.001; r=0.488). On the other hand, expression of this chemokine was positively correlated with the infiltration of CD4+ lymphocytes into the tumor microenvironment (n=67; p=0.019; r=0.286) and with total count of lymphocytes

(n=67; p=0.025; r=0.273). Infiltration of cytotoxic T-lymphocytes (CD8) or regulatory T cells (CD4/FoxP3) was not correlated with CCL2 epithelial expression or with the infiltration of CCL2+ macrophages.

Expression of FPN1 in stromal inflammatory cells

We next analyzed FPN1 expression, by immunohistochemistry, in stromal inflammatory cells. Regarding macrophages, considering pure lesions, FPN1 was higher in pure DCIS lesions. Kruskal-Wallis test was performed and statistically significant differences were found between sub-groups of pure lesions ($p<0.001$). According to the Dunn-Bonferroni correction, differences in FPN1 expression in macrophages were statistically significant when comparing normal samples to pure DCIS ($p=0.001$) and pure DCIS to pure IDC ($p<0.001$). When considering the non-neoplastic tissue adjacent to carcinomas, differences in FPN1 expression in macrophages were statistically significant ($p=0.009$). According to the Dunn-Bonferroni test, differences between sub-groups of lesions were only statistically significant when comparing normal tissue to non-neoplastic tissue adjacent to DCIS lesions ($p=0.007$). In lymphocytes, according to the Kruskal-Wallis test differences between sub-groups of pure lesions were statistically significant ($p=0.011$). Dunn-Bonferroni correction revealed differences statistically significant when comparing normal samples to pure IDC ($p=0.016$). In addition, no statistically significant differences were found between normal tissue from reduction mammoplasties and non-neoplastic tissue adjacent to carcinomas, regarding FPN1 expression in lymphocytes ($p=0.079$).

Association of CCL2 expression and tissue iron deposition

We next determined whether CCL2 expression was associated with tissue iron status and explored this association. Samples presenting iron deposition in epithelial cells showed similar median CCL2 levels to samples without iron deposits. On the other hand, epithelial CCL2 expression was associated with iron deposition in stromal inflammatory cells (n=69; $p=0.001$). In the presence of hemosiderin deposits in lymphocytes and macrophages, median CCL2 expression in epithelial cells was superior, comparing to samples without iron deposition in stromal inflammatory cells.

Association of CCL2 and FPN1 expression

The remarkable increase in FPN1 expression in stromal inflammatory cells, in carcinomas, and the association obtained between CCL2 expression and iron status, led us to analyze whether CCL2 and FPN1 expression could be correlated. Epithelial CCL2 expression was correlated with FPN1 expression in lymphocytes (n=55; $p=0.001$; $r=0.428$), but not in macrophages.

Association of FPN1 expression with tissue iron status

Next, we analyzed whether the increased FPN1 expression observed previously was due to increased iron levels in stromal inflammatory cells. In macrophages and lymphocytes, FPN1 expression was not correlated with iron deposition in stromal inflammatory cells.

Influence of HFE variants in CCL2 expression

Considering that polymorphisms in the HFE gene are relatively common in the general population and given the fact that they were described as modifiers of CCL2 expression, we next analyzed whether the presence of p.C282Y and p.H63D variants could influence the expression of this chemokine. Of the 83 samples studied, genotyping of the p.C282Y variant was possible for 76 individuals, of which 9 were heterozygous and the remaining homozygous wild type. Genotyping for the p.H63D variant was possible for 69 individuals, of which 36 were homozygous wild type, 27 were heterozygous and 2 were homozygous for the variant. In this study, given the low frequency of individuals with the p.C282Y variant, further statistical tests were not performed. CCL2 expression in epithelial cells was not associated with the HFE H63D variant.

Correlation of CCL2 expression with clinico-pathological parameters

We next determined whether epithelial CCL2 expression was associated with clinical-pathological parameters of breast cancer behavior and prognosis. CCL2 expression was significantly higher in epithelial cells of ER-negative DCIS cases (n=25; p=0.011). In invasive ductal carcinoma samples, CCL2 expression in epithelial cells was not associated with the estrogen receptor status. Statistical analysis did not show significant associations between epithelial CCL2 expression and classical prognostic parameters, such as tumor size (n=35; p=0.249), molecular subtype (n=55; p=0.215) and lymph node involvement (n=35; p=0.817).

Discussion

With the present study, we shed new light into the role of stromal inflammatory cells and CCL2, one of the chemokines responsible for their attraction into the tumor microenvironment. A large body of evidence points to the fact that cells in the tumor microenvironment also undergo alterations in response to stimuli sent by epithelial cells, consequently contributing to tumor progression (29). Furthermore, data from different fields of research suggest that in current cancer biology, it should be virtually impossible to address the importance of the tumor milieu without considering the nutritional role of

iron (30). Recent work performed by our group demonstrated that tumor infiltrating macrophages and lymphocytes display an “iron-donor” phenotype, which suggests that they may act as an iron reservoir, hypothetically contributing to tumor nutrition (18). Results herein described corroborate the hypothesis that stromal inflammatory cells may play a fundamental role in tumor progression, demonstrated by the increased expression of FPN1 in these cells independently of iron levels. In this study, a higher percentage of breast cancer samples presented hemosiderin deposits in epithelial tumor cells, in comparison to normal samples. Due to their incessant proliferation, neoplastic cells have high nutritional requirements, which results in homeostatic deregulation favoring higher intracellular concentrations of iron (9). As first described by Pinnix and colleagues, the deregulation of the ferroportin/hepcidin axis may be central in breast tumor progression (8). Moreover, Chen and co-workers, validating a previous work by Zhang and co-workers (11), reported that FPN1 reduction in epithelial cells was associated with increased intracellular iron levels, which suggests that actively proliferating neoplastic cells have a constant supply of iron, necessary for metabolic reactions (31). Remarkably, we detected increased iron deposition not only in the established malignant lesions, but also in the hypothetically normal tissue adjacent to the representative breast lesion. These results suggest that the non-neoplastic tissue adjacent to carcinomas already present alterations that predispose the microenvironment to acquire more iron for metabolic processes. So, iron deregulation may occur previously to the establishment of lesions and is already present in non-malignant lesions, such as hyperplasias, therefore facilitating epithelial cell proliferation, dysplasia and the potential accumulation of mutations (1).

Our results also demonstrate that increased iron deposition is not limited to epithelial cells and that lymphocytes and/or macrophages, especially in carcinoma samples, also present iron accumulation. Lymphocytes are capable of uptaking non-transferrin-bound iron, as demonstrated in a recent study by Pinto and colleagues (32). Macrophages are also specialized cells in handling iron (16) and the regulation of iron homeostasis performed by these cells is possibly not restricted to the systemic level, but also occurs locally. In response to the presence of heme, as a consequence of erythrophagocytosis, FPN1 expression in macrophages is upregulated, independently of hepcidin (33). Based on several evidences, de Sousa hypothesized that in situations of increased blood flow and in the presence of heme, namely in angiogenesis, macrophages mimic this process, by delivering iron to facilitate tumor growth (30). As suggested by de Sousa (30) and supported by the studies previously presented, circulating immune cells may have a surveillance role in controlling potential iron toxicity (34). Tumor-associated macrophages, characterized as M2-like macrophages, act as iron-deficient and are

characterized by higher FPN1 expression. Recalcati and colleagues reported that M2 macrophages are capable of exporting iron *in vitro*, which may exacerbate the neoplastic disease by supplying iron to actively proliferating tumor cells (35). As also reported in the work here presented, increased FPN1 expression in lymphocytes and macrophages, particularly in the "normal" to ductal carcinoma *in situ* transition, may reinforce the iron exporting phenotype suggested for stromal inflammatory cells.

Chemokines are best known for their ability to induce cell migration and significantly contribute to cancer progression and metastasis (36). Taking this into consideration, expression of CCL2 in epithelial and stromal inflammatory cells was analyzed by immunohistochemistry. In this study, an increased expression of CCL2 in epithelial and stromal inflammatory cells was observed with increasing malignancy.

Mantovani was the first to report that tumor-derived chemokines could be responsible for the attraction of monocytes into the tumor nest, where they could enhance tumor progression, by supplying angiogenic factors and promoting growth (37). The evidence of a significant association between the expression of epithelial CCL2 and the infiltration of CCL2-positive macrophages consolidates the idea of a paracrine signaling pathway. Several authors demonstrated the existence of this pathway, in which tumor cells produce CCL2, responsible for the egress of CCR2-positive monocytes from the bone marrow into the tumor area (38). Tissue macrophages also secrete CCL2, recruiting more macrophages, as demonstrated by Fujimoto and colleagues (39). Our study demonstrates that the infiltration of CCL2-positive macrophages was positively associated with total macrophage infiltration. These results suggest that, as proposed by Fujimoto and colleagues, CCL2 secreted by macrophages, attracted by tumor secreted CCL2, is accountable for the attraction of macrophages from the bone marrow into the breast tumor milieu (39).

Epithelial expression of CCL2 was also positively correlated with the infiltration of $CD4^+$ lymphocytes and total lymphocyte infiltration. Although CCL2 is considered to be a monocyte chemoattractant, some studies report that this chemokine may also be an agonist for lymphocytes. Studies performed by Owen and colleagues reported co-localization of the CCR2, the CCL2 receptor, and the pan T cell marker CD3 (40). Moreover, results from a study using a melanoma cell line incubated into mice, suggest that the migration of lymphocytes is dependent on CCL2 produced by tumor cells (41).

The influence of iron status in CCL2 expression was already demonstrated by Mitchell and colleagues (21) and in other studies not related to neoplastic disease (22-25). In our study, the presence of iron deposits in stromal inflammatory cells was positively

associated with increased epithelial CCL2 expression. This significant association suggests iron as a putative driving force to enhance CCL2 expression in the breast tumor environment.

FPN1 expression can be regulated at the transcriptional (by iron deficiency hypoxia, heme and inflammatory cytokines), post-transcriptional (by the IRP-IRE system) or post-translational levels (by hepcidin) (42). Delaby and colleagues demonstrated that FPN1 expression in macrophages and lymphocytes was mainly regulated by iron levels, putatively by the IRP-IRE system (33). In our study FPN1 post-transcription is not regulated by iron levels, suggesting the existence of an alternative signal in the regulation of the iron exporter in breast cancer. Furthermore, expression of FPN1 in lymphocytes, which had been shown to be independent from the iron deposition in stromal inflammatory cells or hepcidin regulation (data not shown), was significantly correlated with CCL2 expression in epithelial cells.

These results led us to propose the existence of a mechanism, in which epithelial cells produce CCL2, whose expression may be putatively enhanced by increased iron deposition in stromal inflammatory cells, resulting in the attraction of circulating macrophages and lymphocytes into the tumor site. When these immune cells arrive to the local, FPN1 expression is upregulated, at least partially by the expression of CCL2, and iron is supplied to malignant cells. CCR2-positive macrophages, attracted to the tumor milieu, are responsible for the production of more CCL2, resulting in more infiltrating stromal inflammatory cells (Figure 6). In conclusion, CCL2 may also play a role in regulating tumor iron nutrition and progression, beyond its function as a chemoattractant.

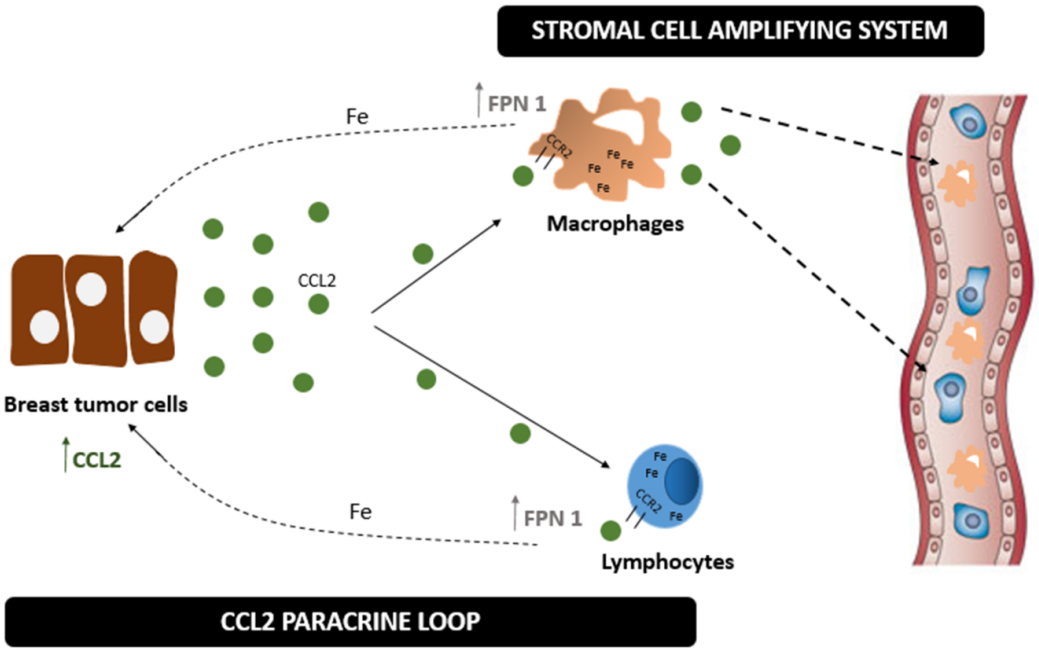

Figure 6. Proposed mechanism of CCL2-induced pathway: CCL2 (green circles), produced by breast tumor cells, attract circulating CCR2-positive cells, such as macrophages and lymphocytes, which are iron-loaded. When these stromal inflammatory cells arrive to the tumor microenvironment, FPN 1 expression is upregulated, partially by the expression of CCL2, and iron (Fe) is supplied for proliferating epithelial cells. Iron supplied by macrophages and lymphocytes leads to increased proliferation and CCL2 secretion by tumor cells, triggering the paracrine signalling pathway, between tumor and immune cells. Macrophages are also accountable for the production of CCL2, resulting in increased leukocyte infiltration and increased iron supply for proliferating neoplastic cells.

Conflicts of Interest

The authors declare that they have no conflict of interest.

Acknowledgments

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Author Details

Conceived and designed the experiments: AMR, OM, CL, GP. Performed the experiments: AMR, OM, LL, ACL and AR. Analyzed the data: AMR, OM, PF, GP, BMS and CL. Contributed with reagents, materials and analysis tools: OM, ACP, PF, BMS and CL. Wrote the paper: AMR, OM, GP. Revised the paper: GP, BMS and CL. All authors read and approved the final manuscript.

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Chapter 5

HFE and the Expression of Iron-Related Proteins in Breast Cancer

HFE VARIANTS AND THE EXPRESSION OF IRON-RELATED PROTEINS IN BREAST CANCER INFLAMMATORY STROMAL CELLS

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Keywords: breast cancer; HFE; iron

Abstract

The association of HFE major variants with breast cancer risk and behavior has been a matter of discussion for a long time. However, their impact on the expression of iron-related proteins in the breast cancer tissue has never been adressed. In the present study, hepcidin, ferroportin 1, transferrin receptor (TFR1), ferritin expression and tissue iron deposition were evaluated in a sample collection of invasive breast cancers according to the patients' HFE genotype. Heterozygous patients for the p.C282Y variant presented a higher expression of hepcidin in lymphocytes and macrophages than wild-type or single p.H63D carrier IDC patients. An increased expression of TFR1 in all the cell types analyzed was also observed in p.C282Y/H63D compound heterozygotes, only. A differential impact of the two HFE variants was further noticed with the observation of a significantly higher percentage of p.C282Y heterozygous patients presenting tissue iron deposition in comparison to p.H63D heterozygotes. No significant associations were found in this study between HFE variants and the classical clinicopathological markers of breast cancer behavior and prognosis. Although limited by a low sampling size, our results suggest that HFE major variants could play a role in breast cancer progression not by influencing systemic iron homeostasis but rather by differentially modulating the local cellular expression of iron-related proteins and tissue iron deposition.

Introduction

Iron is an essential trace element for the human body, as a critical component of several signaling, transporter and storage molecules involved in energy production and intermediate metabolism (Andrews, 1999; Ganz and Nemeth, 2011). However, its characteristic chemistry contributes to the formation of hazardous molecules, such as hydroxyl radicals and hydrogen peroxide, when in excess (Fenton, 1894; Halliwell and Gutteridge, 1992; Kalinowski and Richardson, 2005). Although most organisms have the proper mechanisms to regulate iron homeostasis to avoid free iron toxicity, current knowledge suggests that the deregulation of its regulatory mechanisms may contribute to a number of chronic diseases (Kell, 2009). Iron is thought to promote carcinogenesis through iron-induced oxidative stress, modulation of signaling networks associated with malignancy and by providing selective advantage to rapidly growing tumor cells (Omary et al., 1980; Cermak et al., 1993; Eaton and Qian, 2002; Benhar et al., 2002; Kowdley, 2004; Galaris et al., 2008).

HFE is a MHC class-I like protein that acts as a gatekeeper of systemic iron homeostasis by controlling hepatic hepcidin levels (Schmidt et al., 2008; Vujic Spasic et al., 2008). Hepcidin, in turn, maintains normal plasma iron levels by regulating iron release from cells through the binding to its receptor, the iron exporter ferroportin 1 (Nemeth et al., 2004; Nemeth and Ganz, 2006). A proposed molecular mechanism positions HFE and Transferrin Receptor 1 (TFR1) in an iron-sensing complex which is disrupted by binding of circulating holotransferrin with a higher affinity for TFR1 (Goswami and Andrews, 2006). Upon TFR1 dissociation, HFE is able to relocate to TFR2 and interact with the bone morphogenetic protein (Bmp) co-receptor Hemojuvelin (Goswami and Andrews, 2006; D'Alessio et al., 2012), involved in signal communication upon binding of the Bmp ligands, and whose interaction leads to the activation of hepcidin transcription (Miyazono et al., 2005; Babitt et al., 2006; Babitt et al., 2007). However, previous evidences from others suggest that HFE may also act a regulator of iron uptake through its direct interaction with the TFR1 (Feder et al., 1996b; Lebron et al., 1998; Waheed et al., 2002). HFE gene variants p.C282Y and p.H63D are very common in normal European derived populations. The p.C282Y variant disrupts the association of HFE with β-2 microglobulin, reducing the cellular surface expression of HFE (Feder et al. , 1996b; Feder et al., 1997; Waheed et al., 1997). This alteration is responsible for the large majority of hereditary hemochromatosis cases (Feder et al. , 1996b). The p.H63D variant is believed to lower the HFE protein affinity for TFR1 (Gray et al., 2009), but its association with iron overload is controversial (Aguilar-Martinez et al., 2001; Kelley et al., 2014; Porto et al., 2015). Although epidemiological studies have been inconsistent in supporting an association between HFE

major variants and an increased risk for breast cancer (Nelson et al., 1995; Beckman et al., 1999; Kallianpur et al., 2004; Abraham et al., 2005; Osborne et al., 2010), it is plausible to assume that, by interfering with the cellular and tissue iron homeostasis, they may affect the cancer cell phenotype.

We have previously shown that the deregulation of iron-related proteins in breast cancer, more specifically hepcidin, ferroportin 1 (FPN1), TFR1 and ferritin (FT), is not restricted to epithelial cells, but also extends to cells of the tumor microenvironment (Marques et al., 2016). To our knowledge, no other group has attempted to verify if the HFE major variants had an impact on the expression of iron-related proteins in the neoplastic context.

Materials and Methods

Sample Characterization

A previously characterized cohort of human breast tissue samples, archived at the Pathology Service at Centro Hospitalar do Porto, was used in this study. This cohort consisted of 120 samples, including 56 cases of invasive ductal carcinomas (IDC), 14 cases of ductal carcinomas *in situ* (DCIS) and 49 samples without evidence of breast disease obtained from breast reduction aesthetic surgery. The study has been previously approved by the local Research Ethics Committees, as part of a more extended study (see Marques et al., 2016). Clinicopathological features, such as histological diagnosis, estrogen receptor (ER), progesterone receptor (PR) and human epidermal growth factor 2 (HER2) statuses were retrieved from interin pathology reports. ER, PR and HER-2 status were assessed by immunohistochemistry. HER-2 ambiguous results were confirmed by FISH.

Tissue Microarray Construction and Immunohistochemistry

Tissue microarray construction and immunohistochemistry for hepcidin, FPN1, TFR1 and FT for this cohort have been extensively described before (Marques et al., 2016). Immunostaining for hepcidin, FPN1, TFR1 and FT was evaluated in epithelial cells, lymphocytes and macrophages using the same semi-quantitative evaluation method as before (Marques et al., 2016). Cores from the same donor tissue were grouped and their mean score for each variable calculated.

DAB-enhanced Perls' Prussian Blue Staining

To evaluate the presence of iron deposition in breast samples, DAB-enhanced Perls' Prussian Blue was performed, adapted to the Van Duijn protocol (Van Duijn et al., 2013).

DNA Extraction and HFE Genotyping

Genomic DNA was extracted from FFPE breast sections according to the Ultraprep Tissue DNA kit (AHN Biotechnologie, Nordhausen, Germany) recommended procedures. PCR was carried out in 15.5μL reaction volumes, containing 2μL of the genomic DNA template, 7.5μL of MasterMix DNA polymerase, 1μL of Q-solution (Qiagen Multiplex PCR kit, Valencia, CA, USA) and 1μL of each of sense and antisense primers. For the detection of the p.C282Y variant the following primers were used: 5'-CAAGTGCCTCCTTTGGTGAAGGTGACACAT-3' as the forward primer and 5'-CTCAGGCACTCCTCTCAACC-3' as the reverse primer (Metabion, Steinkirchen, Germany). Following the verification of the 343 bp fragment amplification RsaI was used for restriction. For the HFE p.H63D variant, the following forward and reverse primers' sequences were used: 5'-ACA TGG TTA AGG CCT GTT GC-3' and 5'-GCC ACA TCT GGC TTA AAA TT-3' (Metabion, Steinkirchen, Germany). In turn, these primers amplify a fragment with 294 bp that was then restricted by MboI. Primers for detection of variants in the HFE gene were chosen according to the work of Feder *et al.* (Feder et al., 1996a). The PCR program included a step of 95ºC, for 15 minutes followed by 36 cycles of denaturation at 94ºC for 30 seconds, annealing at 58ºC for 90 seconds and extension at 72ºC for 90 seconds. Reaction was extended in the final for 10 minutes at 72ºC.

Statistical Analysis

Data were analyzed with IBM SPSS Statistics Version 18.0 (SPSS Inc., IBM, Chicago, IL, USA). Sample distributions were compared by the Kruskal-Wallis test followed by post-hoc testing whenever the omnibus testing was significant. Pearson's Chi-Square was used to evaluate the differences between categorical variables. In figures, experimental errors are shown as 95% Confidence Intervals (CI). Statistical significance was accepted at $p < 0.05$.

Once no p.C282Y heterozygotes were found within the aesthetic breast reduction population, comparisons for this variant were restricted to breast cancer cases.

Results

Expression of Iron-Related Proteins

No significant differences were found in the expression of the analyzed iron-related proteins between wild-type individuals and p.H63D carriers included in the aesthetic breast reduction population for all cell types. Similarly, no significant differences were found for the expression of these proteins in DCIS among all the genotypes considered. In invasive breast carcinoma (IDC) cases, however, the expression of hepcidin in lymphocytes and macrophages was significantly higher in patients carrying the p.C282Y variant ($p < 0.05$; Figure 1A) relative to both wild type and p.H63D carriers. No further differences were found regarding the expression of FPN1, TFR1 and FT in IDC cases (Figure 1B-D).

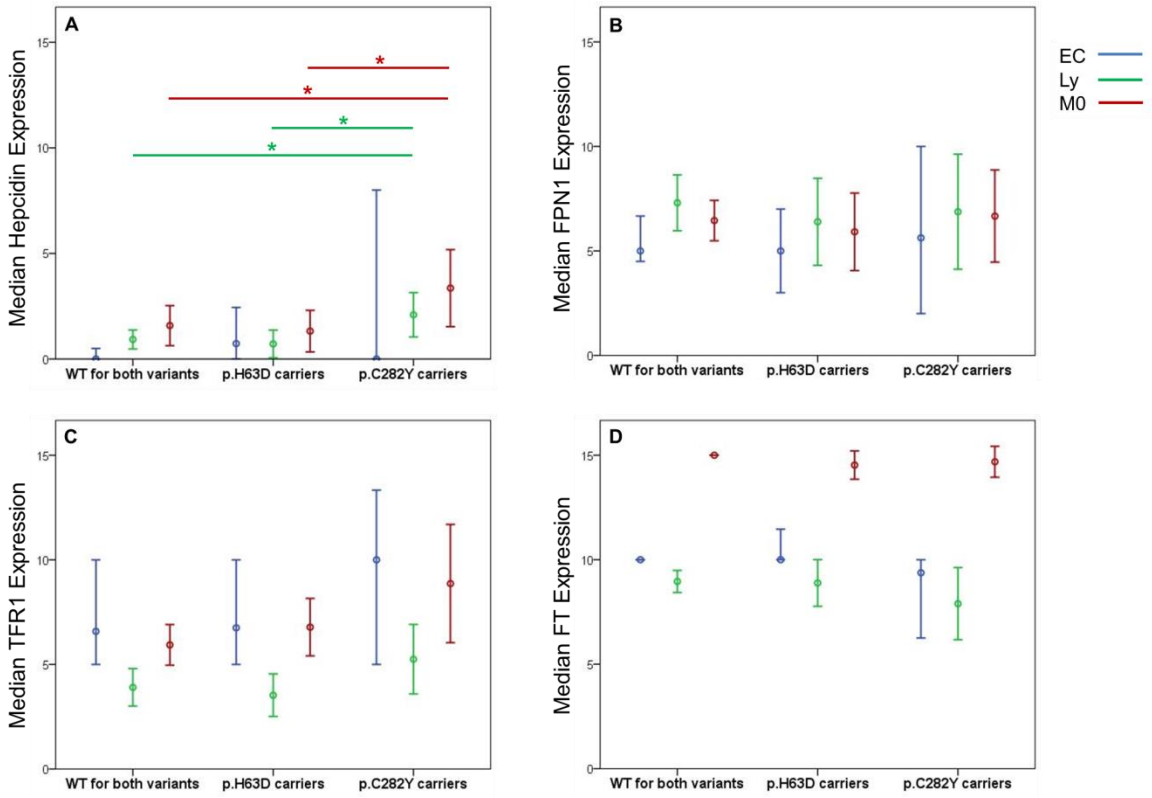

Figure 1. Iron-related proteins in invasive breast carcinomas according to HFE genotype. Median hepcidin (A), FPN1 (B), TFR1 (C) and FT (D) expression, in epithelial cells, lymphocytes and macrophages, in relation to the presence or absence (WT) of the p.C282Y and p.H63D variants. Scores ranged from 0 to 15 and errors bars present 95% CI. Abbreviations: WT, Wild-Type; FPN1, ferroportin 1; TFR1, transferrin receptor 1; FT, ferritin; EC, Epithelial Cells; Ly, Lymphocytes; M0, Macrophages

To further clarify the relative impact of the two variants in the results observed, we focused the analysis on a sub-sample of p.C282Y/p.H63D compound heterozygotes. Remarkably, from the 9 p.C282Y carriers with IDC, 5 were compound heterozygotes. Although these did not differ in general from the other p.C282Y/WT IDC patients in terms of the expression of the iron-related proteins, they differed significantly from non-p.C282Y carriers not only by a higher expression of hepcidin in lymphocytes and macrophages (as described in the whole p.C282Y carrier population) but they showed, in addition, an increased expression of TFR1 in all the cell types analyzed (Table 1).

Table 1. Expression of iron-related proteins in HFE p.C282Y/p.H63D compound heterozygous IDC patients is increased in comparison with patients without the p.C282Y variant. * represents significant differences (p< 0.05) for comparison with the non-p.C282Y carriers group. Abbreviations: IDC, invasive ductal carcinoma; SEM, Standard Error of the Mean; EC, Epithelial Cells; Ly, Lymphocytes; M0, Macrophages; NS, Non-Significant

Iron-Related Proteins (Mean ± SEM)	non-p.C282Y carriers (n= 47)	p.C282Y/p.H63D heterozygous compounds (n= 5)	p.C282/WT heterozygous (n= 4)
Hepcidin			
EC	1.06 ± 0.28	3.4 ± 2.93	6.38 ± 3.33
Ly	0.80 ± 0.16	2.17 ± 0.69*	2.00 ± 0.58
M0	1.56 ± 0.32	3.60 ± 0.98*****	3.83 ± 1.30
FPN1			
EC	5.54 ± 0.39	6.11 ± 1.02	6.13 ± 1.71
Ly	6.67 ± 0.54	8.43 ± 1.06	4.28 ± 1.88
M0	6.17 ± 0.43	7.90 ± 0.75	4.61 ± 1.69
TFR1			
EC	7.25 ± 0.51	11.00 ± 1.72*****	7.65 ± 1.98
Ly	3.70 ± 0.32	5.80 ± 0.97*****	4.33 ± 0.88
M0	6.14 ± 0.38	9.68 ± 1.42*****	7.50 ± 2.25
FT			
EC	10.09 ± 0.35	8.92 ± 0.74	9.06 ± 0.60
Ly	8.89 ± 0.24	8.50 ± 1.07	6.88 ± 0.59*****
M0	14.83 ± 0.12	15.00 ± 0.00	14.17 ± 0.83

Tissue Iron Deposition

Regarding tissue iron deposition in invasive breast carcinomas, a significantly lower percentage of p.H63D carrier IDC patients presented iron deposits in epithelial and stromal inflammatory cells, compared with the two other genotypes considered ($p < 0.05$; Figure 2). Differences between wild-type and p.C282Y heterozygotes were not statistically significant. From the p.C282Y carrier IDC patients displaying iron deposits in epithelial cells, all of them were compound heterozygotes and from the ones with iron deposition in stromal inflammatory cells, 4 out of 5 were compound heterozygotes.

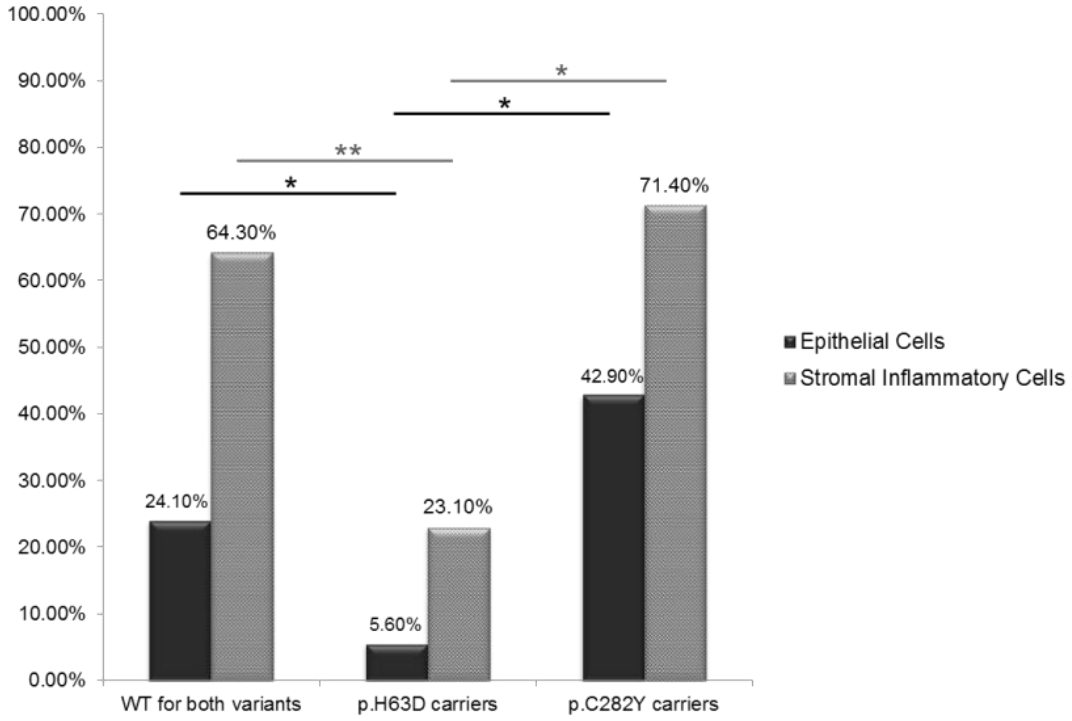

Figure 2. Iron deposition in invasive breast carcinomas according to the HFE genotype. Percentage of invasive breast cancer cases presenting iron deposition in epithelial (black) and stromal inflammatory (grey) cells, in relation to the HFE genotype. Abbreviations: WT, Wild-Type

Clinicopathological Data

Hormone receptor and HER2 status, lymph node involvement and tumor size were available from the interin records and were also analyzed regarding the HFE genotype. None of the different genotypes were associated with any of the considered variables of poor outcome (Table 2).

Table 2. Clinicopathological features of breast cancer patients according to their HFE genotype. Abbreviations: DCIS, ductal carcinoma *in situ*; IDC, invasive ductal carcinoma; ER, estrogen receptor; PR, progesterone receptor, HER2, Human Epidermal growth factor Receptor 2; LN, lymph-node; IQR, interquartile range; NS, non-significant

Factors	DCIS				IDC			
	WT n (%)	p.H63D carriers n (%)	p.C282Y carriers n (%)	*p*	WT n (%)	p.H63D carriers n (%)	p.C282Y carriers n (%)	*p*
ER status, n (%)[a]								
negative	2 (28.6%)	1 (25.0%)	2 (66.7%)	NS	7 (22.6%)	3 (16.7%)	3 (37.5%)	NS
positive	5 (71.4%)	3 (75.0%)	1 (33.3%)		24 (77.4%)	15 (83.3%)	5 (62.5%)	
PR status, n (%)[a]								
negative	3 (42.9%)	2 (50.0%)	2 (66.7%)	NS	8 (25.8%)	5 (27.8%)	4 (50.0%)	NS
positive	4 (57.1%)	2 (50.0%)	1 (33.3%)		23 (74.2%)	13 (72.2%)	4 (50.0%)	
HER2 status, n (%)[a]								
negative	4 (57.1%)	1 (33.3%)	2 (66.7%)	NS	24 (77.7%)	13 (76.5%)	4 (50.00%)	NS
positive	3 (42.9%)	2 (66.7%)	1 (33.3%)		7 (22.6%)	4 (23.5%)	4 (50.00%)	
LN metastasis, n (%)[a]								
non-metastized					17 (56.7%)	4 (22.2%)	4 (50.0%)	NS
metastized					13 (43.3%)	14 (77.8%)	4 (50.0%)	
Median tumor size (IQR)[b]					1.00 (1.00 - 2.00)	1.00 (1.00 - 2.00)	1.00 (1.00 - 1.50)	NS

[a] Pearson Chi-Square

[b] Kruskal Wallis Test

Discussion

The fact that HFE variants are much more prevalent than the iron overload disorder hereditary hemochromatosis (Waalen et al., 2005) contributed to an increasing interest in the HFE gene as a risk factor or disease modifier in various chronic diseases, such as cancer. However, genetic association studies have not been successful in demonstrating a clear relationship between HFE major variants and increased breast cancer risk (Nelson et al., 1995; Beckman et al., 1999; Abraham et al., 2005; Osborne et al., 2010).

In our study, we describe a higher expression of hepcidin in lymphocytes and macrophages of IDC patients with the p.C282Y variant (Figure 1A, Table 1). Although other studies suggest that, in general, hepcidin expression does not differ significantly from that of healthy subjects (Roe et al., 2007), we cannot ignore the local regulation and the possibility that an association between HFE variants and other proteins, such as p16, may be cell-specific or even restricted to neoplastic context (Lee et al., 2011). Furthermore, p.C282Y/p.H63D compound heterozygous IDC patients display a more drastic phenotype by enhanced expression of TFR1 in all the cell types analyzed. A study by Piperno and colleagues has demonstrated that, at the time of diagnosis, while p.C282Y homozygous HFE patients presented comparable hepcidin levels to controls, compound heterozygotes displayed increased values (Piperno et al., 2007). Furthermore, the p.H63D variant, in association with the p.C282Y has been associated with increased hepatic iron concentrations (Bacon et al., 1999; Brissot et al., 1999). Particularly, in a study with healthy women, increased serum iron levels and transferrin saturation were observed in compound heterozygotes, but not in p.C282Y/WT, when compared with women with none of the variants (Rossi et al., 2000). The increased iron deposition in epithelial and stromal inflammatory cells of IDC patients with the p.C282Y variant may, thus, be a consequence of not only increased iron retention due to the local hepcidin effect but also due to increased TFR1 expression as a result of neoplastic epithelial cells' 'iron-deficient' phenotype (Pinnix et al., 2010; Wang et al., 2014; Zhang et al., 2014) and stromal inflammatory cells' activation (Manger et al., 1986; Paulnock and Lambert, 1990).

The fact that only differences in IDC patients were found may partially explain the inconsistent results trying to relate the p.C282Y variant and breast cancer risk. Although others have observed an increased prevalence of the p.C282Y variant with a higher number of lymph-nodes affected (Abraham et al. , 2005) we have not found any association between classic clinicopathological markers in breast cancer and any of the HFE genotypes considered. Although a slightly higher prevalence of the p.C282Y variant

was observed in patients with markers of poor outcome (ER-, PR-, HER2+), the insufficient sampling size does not allow us to draw further conclusions.

In conclusion, in spite of the limitation of this study due to low population numbers, the results give further support to the concept of an alternative role for HFE in chronic diseases through modulation of local iron homeostasis, and highlights the need to a more insightful knowledge of the role of HFE in cancer.

Conflicts of Interest

The authors declare they have no competing interests.

Author Contributions

Conceived and designed the experiments: OM, PF, BMS, GP and CL. Performed the experiments: OM, AR, LL and AR. Analyzed the data: OM, PF, BMS, GP and CL. Contributed with reagents, materials and analysis tools: PF, BMS, GP and CL. Wrote the paper: OM and GP. All authors revised and approved the final manuscript.

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Chapter 6

Expression of Iron-Related Proteins in Feline and Canine Mammary Gland Reveals Unexpected Accumulation of Iron

EXPRESSION OF IRON-RELATED PROTEINS IN FELINE AND CANINE MAMMARY GLAND REVEALS UNEXPECTED ACCUMULATION OF IRON

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Abstract

Breast cancer is characterized by a deregulation in cellular iron homeostasis, as reflected by the altered expression of its regulatory proteins. Malignant epithelial cells present higher iron requirements, behaving as 'iron-deficient', while stromal inflammatory cells may act as 'iron-donors'. The expression of iron-related proteins in the mammary gland of cats and dogs has never been assessed before. We evaluated the expression of Hepcidin, Ferroportin 1 (FPN1), Transferrin Receptor 1 (TFR1) and Ferritin (FT) in epithelial cells, lymphocytes and macrophages of cats and dogs' benign and malignant mammary gland lesions. Iron deposition was detected with Perls' Prussian Blue staining. Unlike human breast cancer, no major differences were found in the expression of the iron-related proteins between benign and malignant mammary gland lesions from cats and dogs. However, cats and dogs show accumulation of iron in benign lesions, demonstrated by the high percentage of samples presenting hemosiderin deposition, thus providing an explanation for the absence of higher iron requirements by tumor cells. Although a role for iron-induced chronic oxidative stress cannot be excluded, physiological differences in relation to human breast cancer demand a more profound knowledge of local iron homeostasis in cats and dogs.

Introduction

Breast cancer is the most frequent neoplasia in women worldwide. Although recent advances in early diagnostic and therapeutic regimens have led to lower mortality rates, breast cancer is still by far the most frequent cancer in women and the second most frequent cause of cancer death in developed countries (Ferlay et al., 2013). Likewise, mammary tumors are the most common form of cancer in female intact dogs and the third most frequent in intact cats (Moulton, 1990; Misdorp et al., 1999; Misdorp, 2002; Goldschmidt et al., 2011). Despite livelong discussion about the role of animal models in human research, there is evidence for the similarities between human breast cancer and both canine and feline mammary gland tumors. These include the spontaneous occurrence of tumors and relative early age of onset, hormonal influence, histological features, biological behavior and therapy response (Pang and Argyle, 2009; Queiroga et al., 2011; Rivera and Von Euler, 2011; Matos et al., 2012). Despite this, and aside from BRCA1 and 2 (Rivera et al., 2009), TP53 (Borge et al., 2011) in dogs and HER-2/neu in cats (Millanta et al., 2005; Ordas et al., 2007), transversal molecular targets associated with carcinogenesis remain largely unknown or present inconsistent results. Thus, it remains imperative to pursue further insights in comparative oncology, not only for human research extrapolation but also for the pets' benefit.

One such example is iron's role in breast carcinogenesis. Due to its ability to accept and donate electrons, iron is an essential element and a critical component of sensor, transporter and storing molecules and enzymes involved in energy production and intermediate metabolism (Andrews, 1999; Cairo et al., 2006; Ganz and Nemeth, 2011). At systemic level, hepcidin is responsible for regulating iron absorption, and ultimately iron homeostasis, through its ability to down-modulate the cellular expression of ferroportin 1, the only known iron exporter (Nemeth et al., 2004; Knutson et al., 2005). Cellular iron uptake is regulated by the presence of the transferrin receptor 1 (TFR1), through binding to transferrin and internalization of the iron-loaded complex (Klausner et al., 1983a; Klausner et al., 1983b). To avoid free iron associated toxicity, iron not being used for cellular purposes is stored inside ferritin (FT), an ubiquitous heteropolymer composed of 24 heavy and light subunits (Harrison and Arosio, 1996; Arosio et al., 2009). Comprehensive data supports a role for iron-mediated carcinogenesis (Huang, 2003). Paths proposed include DNA, protein and organelle damage through iron-induced oxidative stress (Nelson, 1992; Toyokuni, 1996; Eaton and Qian, 2002), further activation of signaling pathways associated with oncogenesis (Benhar et al., 2002; Kowdley, 2004; Galaris et al., 2008) and selective advantage for malignant cell clonal expansion (Omary et al., 1980; Cermak et al., 1993). Particularly, studies in animal models have consistently

demonstrated that subcutaneously injected iron and the administration of iron-rich diets promotes mammary gland tumor development and progression (Thompson et al., 1991; Singh et al., 1994; Hrabinski et al., 1995; Diwan et al., 1997).

Although the malignant state in human breast cancer cells has been characterized by a deregulation in cellular iron homeostasis, and consequently in the expression of iron-regulatory proteins, this issue has never been addressed in canine and feline mammary tumors. In the present study we analyzed the expression of iron-regulatory proteins hepcidin, ferroportin 1, transferrin receptor 1 and ferritin in both canine and feline mammary epithelial cells, lymphocytes and macrophages from benign and malignant lesions. This approach allowed us to conclude that the expression of these iron-related proteins does not differ from benign to malignant lesions from cats and dogs, because there is already an evident strong iron accumulation in normal mammary gland tissues.

Materials and Methods

Sample characterization

Paraffin-embedded blocks from 68 cats and 62 dogs archived in the Veterinary Pathology Laboratory (ICBAS) and the Histology and Anatomical Pathology Laboratory (UTAD) from 2001 to 2011 were included in this study. From the sampled animals, 59 cats presented carcinomas and 9 had benign alterations (including fibroadenomatous alterations and hyperplasias). Regarding the dogs, 36 presented carcinomas and 26 benign lesions (including hyperplasias, adenomas and benign mixed mammary tumors). All mammary lesions were histologically classified according to the criteria defined by the World Health Organization (WHO) for the classification of dog and cat mammary tumors by three veterinary pathologists (AC, FS and AG). Histological grading of tissue samples was performed through a modified Elston and Ellis method (Elston and Ellis, 1991), by assessment of the degree of tubular formation, nuclear polymorphism and mitotic activity, in 5 high power fields, as previously described for cats (Seixas et al., 2011) and dogs (Clemente et al., 2010).

Tissue Microarray construction

The correspondent formalin-fixed paraffin-embedded (FFPE) tissue blocks and their corresponding hematoxylin and eosin (H&E)-stained slides were retrieved from the archives and carefully analyzed by a pathologist (CL, FS and AG) as to select target areas. Whenever possible, non-malignant lesions were selected from carcinoma cases, to ensure tissue representativity. Sections of human liver were obtained from the archive of

the Pathology Department of Santo António Hospital, Porto Hospital Centre, and were included in each tissue microarray block, as positive controls.

Immunohistochemistry

Immunohistochemistry was performed on 2 µm tissue microarray paraffin-embedded sections with the following antibodies; hepcidin (dilution 1/500, Abcam, Cambridge, UK), ferroportin 1 (FPN1 - 1/750, Novus Biologicals Europe, Cambridge, UK), CD71 (TFR1 [clone 10F11] - 1/80, Novocastra, Newcastle, UK) and ferritin (FT - 1/1000, Sigma-Aldrich, MO, USA). Briefly, sections were deparaffinized in xylene and rehydrated in decreasing concentration of ethanol and washed in water, for 5 minutes each. Heat-mediated antigen target retrieval was done with DAKOTarget Retrieval Solution (Agilent Technologies, Denmark) in a water-bath, at 99.5ºC, during 25 minutes. Immunohistochemistry was then performed according to Novolink Polymer Detection kit procedures (Leica, Biosystems, Cambridge, UK). Enzyme reactivity was visualized using 3,3′-Diaminobenzidine tetrahydrochloride (DAB, Sigma-Aldrich, MO, USA) and slides were counterstained with Mayers hemalum solution (Merck Millipore, Darmstadt, DE), dehydrated and mounted with Entellan (Merck Millipore, Darmstadt, DE). The reaction product was analyzed in a Leica DM LB microscope. Antibody optimum dilutions were determined in each species' tissue-positive controls (hepcidin – liver, FPN1 – duodenum, TFR1 – tonsil, FT – spleen). Negative controls were performed by replacing the primary antibody with an antibody of the same immunoglobulin isotype, as well as a section of liver tissue from a HAMP KO mouse.

Staining Criteria

Immunostaining for hepcidin, FPN1, TFR1 and FT was evaluated in luminal epithelial cells, lymphocytes and macrophages. A semi-quantitative evaluation method was applied: the percentage of positive cells (0 points: 0%; 1 point: 1-10%, 2 points: 11-20%, 3 points: 21-35%, 4 points: 36-50% and 5 points: >50%) and the staining intensity (0 points: no staining, 1 point: weak staining, 2 points: moderate staining and 3 points: strong staining) were considered and multiplied. Cores from the same donor tissue were grouped and their mean score calculated.

Perls' Prussian Blue staining

Hemosiderin deposits were detected by the routine technique of Perls' Prussian Blue staining. After deparaffinization and rehydration in the ethanol series, 2 µm tissue microarray sections were immersed in a mixture of equal volumes of potassium ferrocyanide solution and hydrochloric acid solution (2%) at 60ºC, during 20 minutes. Counterstaining was achieved with nuclear fast red (Merck Millipore, Darmstadt,

Germany). The absence or presence of hemosiderin deposits was evaluated in epithelial and stromal inflammatory cells.

Electron Microscopy

Mammary gland samples were obtained during necropsy procedures from one dog and one cat who died from non-infectious and non-neoplastic causes. Absence of mammary gland disease was confirmed by routine H&E staining. Mammary gland and liver samples were fixed for about 2 h at 4ºC in 2.5% glutaraldehyde and 4% formaldehyde (obtained from hydrolysis of para-formaldehyde), diluted with cacodylate buffer (0.1 M), pH 7.3. After being washed in the buffer, half the samples were processed with Perls' Prussian Blue *en bloc*, as described previously, but incubated during 40 min. In the other half of the samples this step was omitted. All samples were then postfixed with 2% osmium tetroxide (OsO_4) buffered with cacodylate, dehydrated in increasing concentrations of ethanol and embedded in Epon. In order to identify the areas of hemosiderin deposition, semithin sections from the samples previously subjected to Perls' Prussian Blue reaction *en bloc*, were treated with an ethanol saturated solution of sodium hydroxide to remove the epoxy resin and counterstained with nuclear fast red (Merck Millipore, Darmstadt, Germany). Ultrathin sections, with and without double staining with aqueous uranyl acetate and lead citrate were obtained in JEOL 100CXII transmission electron microscope operated at 60 kV.

Statistical Analysis

Sample distributions were compared using the Mann-Whitney test. Pearson's Chi-Square was used to evaluate the differences between categorical variables. The Spearman's rank correlation coefficient was used to evaluate the relationship between variables. Data were analyzed in IBM SPSS Statistics 20.0 software and statistical significance was accepted at $p< 0.05$.

Results

Iron-related proteins immunoexpression in tissue sections

The immunoexpression of hepcidin, FPN1, TFR1 and FT was assessed in both benign and malignant mammary gland tissue from cats and dogs and the respective results are shown in Table 1.

Table 1. Immunoexpression of iron-related proteins in mammary gland tissue

		Hepcidin (Mean ± SEM[1])	Sig.[2]	FPN1[3] (Mean ± SEM)	Sig.	TFR1[4] (Mean ± SEM)	Sig.	FT[5] (Mean ± SEM)	Sig.
CAT	EC[6]	n= 66		n= 60		n= 66		n= 65	
	Benign Lesion	2.87 (± 0.72)	NS[9]	9.06 (± 0.91)	NS	1.50 (± 0.53)	NS	11.43 (± 0.75)	**p= 0.009**
	Malign Lesion	1.83 (± 0.34)		9.77 (± 0.47)		1.40 (± 0.21)		9.14 (± 0.36)	
	Ly[7]	n= 32		n= 35		n= 54		n= 37	
	Benign Lesion	4.22 (± 2.12)	NS	2.50 (± 0.50)	NS	1.28 (± 0.67)	NS	12.50 (± 0.00)	NS
	Malign Lesion	2.50 (± 0.56)		3.28 (± 0.36)		0.91 (± 0.23)		8.79 (± 0.61)	
	M0[8]	n= 35		n= 37		n= 52		n= 56	
	Benign Lesion	10.33 (± 1.67)	NS	4.50 (± 0.50)	NS	7.67 (± 1.56)	**p= 0.005**	14.69 (± 0.31)	NS
	Malign Lesion	6.88 (± 0.61)		7.41 (± 0.52)		3.29 (± 0.53)		14.90 (± 0.07)	
DOG	EC	n= 60		n= 52		n= 56		n= 57	
	Benign Lesion	1.69 (± 0.27)	NS	10.11 (± 0.74)	NS	2.88 (± 0.51)	NS	10.07 (± 0.53)	NS
	Malign Lesion	1.45 (± 0.29)		10.66(± 0.60)		2.40 (± 0.29)		10.10 (± 0.37)	
	Ly	n= 30		n= 28		n= 47		n= 28	
	Benign Lesion	1.79 (± 0.41)	NS	5.78 (± 1.38)	NS	0.75 (± 1.19)	NS	8.60 (± 1.31)	NS
	Malign Lesion	2.89 (± 0.61)		4.80 (± 0.75)		1.15 (± 0.26)		9.13 (± 0.69)	
	M0	n= 26		n= 33		n= 50		n= 52	
	Benign Lesion	12.04 (± 0.82)	NS	8.72 (± 1.15)	NS	7.57 (± 0.61)	NS	14.77 (± 0.22)	NS
	Malign Lesion	8.93 (± 1.20)		7.72 (± 0.50)		8.02 (± 0.45)		14.92 (± 0.08)	

[1]SEM, Standard Error of the Mean; [2]Sig., Significance; [3]FPN1, Ferroportin 1; [4]TFR1, Transferrin Receptor 1; [5]FT, Ferritin; [6]EC, Epithelial Cells; [7]Ly, Lymphocytes; [8]M0, Macrophages; [9]NS, Non-Significant

Results for individual types of lesions are shown in Supplementary Tables 1 (cat) and 2 (dog). Representative images of the staining patterns are illustrated in Fig. 1.

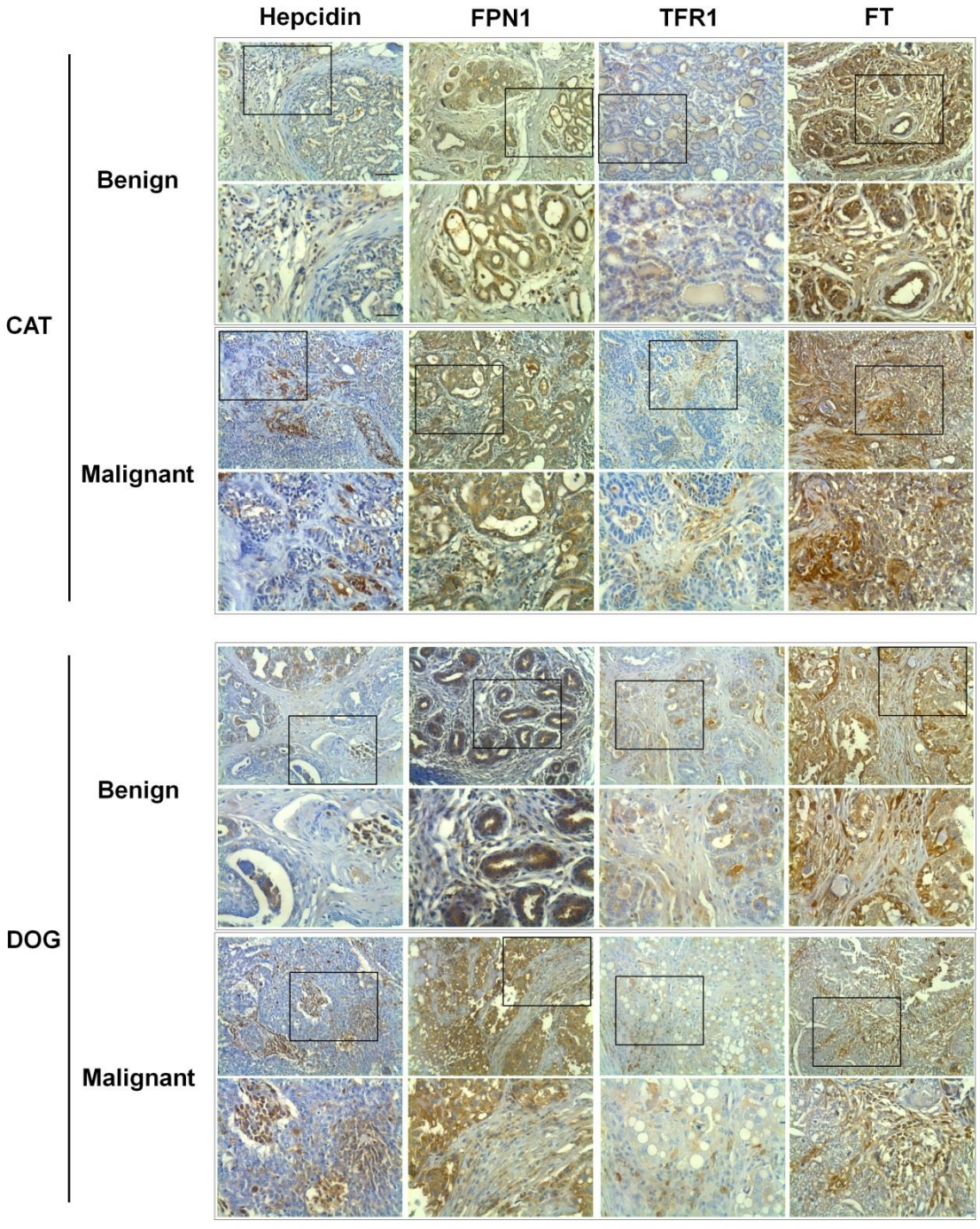

Figure 1. Hepcidin, FPN1, TFR1 and FT expression pattern in the mammary gland tissue of cats and dogs. Representative images of the Hepcidin, FPN1 (Ferroportin 1), TFR1 (Transferrin Receptor 1) and FT (Ferritin) immunostaining in benign and malignant breast lesions. Tissue microarrays containing several samples of cats' and dogs' mammary tissue were constructed, sectioned and subjected to immunohistochemistry, as described in materials and methods. Below each 200× original

magnification image is a 400×-magnification paired image (Scale bar in 200× magnification images - 50 µm; Scale bar in 400× magnification images -25 µm).

Hepcidin expression was observed in the cytoplasm of all the cell types evaluated. No significant differences were observed for the hepcidin expression in epithelial cells, lymphocytes or macrophages between benign and malignant mammary gland tissue of cats and dogs.

Similarly, ferroportin 1 expression was also mainly observed in the cytoplasm of all the cell types assessed, although in some samples, a clear membranous staining in epithelial cells was also noticed. As for hepcidin, no significant differences in FPN1 were detected between benign and malignant mammary gland tissues for the cell types evaluated.

TFR1 expression was predominantly detected in the cytoplasm of every cell type evaluated. However, in some cases membranous staining was also detected in epithelial cells. As for the previous iron-related proteins described no significant differences were observed between benign and malignant mammary gland tissue samples, except for TFR1 expression in macrophages from cats' mammary gland tissue. In this species, macrophages from benign tissues presented a significantly higher expression of TFR1 than in malignant tissues ($p= 0.005$).

Ferritin immunoexpression was mainly observed in the cytoplasm of the cell types assessed. As for the other iron-related proteins, regarding dogs' tissue, no significant differences were found between benign and malignant lesions. However, in cats' mammary gland tissue, a significant higher expression of ferritin in epithelial cells was found in benign lesions, when comparing with malignant ones ($p= 0.009$). Nuclear FT staining in epithelial cells from some samples was noted.

Clinicopathological data

The expression of these iron-related proteins was correlated with grade based on modified schemes of Elston and Ellis classification, currently the most powerful prognostic factor recognized for cats and dogs mammary tumors (Clemente et al., 2010; Seixas et al., 2011). Results are shown in Table 2. FT expression in epithelial cells was negatively associated with increasing tumor grade in cats ($p= 0.017$). All other comparisons, either for cat or dog, were not statistically significant.

Table 2. Correlation table between grade and the expression of iron-related proteins

			Grade		
			n	r	Sig.[1]
CAT	Hepcidin	EC[2]	62	0.058	0.671
		Ly[3]	31	-0.032	0.869
		M0[4]	34	0.248	0.158
	FPN1[5]	EC	58	0.087	0.527
		Ly	33	-0.048	0.793
		M0	33	-0.190	0.290
	TFR1[6]	EC	63	-0.056	0.672
		Ly	53	-0.226	0.111
		M0	51	-0.151	0.307
	FT[7]	EC	**61**	**-0.317**	**0.017**
		Ly	36	-0.220	0.198
		M0	53	-0.080	0.579
DOG	HAMP	EC	60	0.146	0.267
		Ly	30	-0.001	0.997
		M0	36	0.021	0.920
	FPN1	EC	52	0.174	0.216
		Ly	28	0.027	0.891
		M0	33	0.229	0.201
	TFR1	EC	56	-0.143	0.293
		Ly	47	0.063	0.675
		M0	49	0.059	0.687
	FT	EC	56	0.088	0.518
		Ly	28	-0.066	0.737
		M0	52	-0.047	0.740

[1]Sig., Significance; [2]EC, Epithelial Cells, [3]Ly, Lymphocytes; [4]M0, Macrophages; [5]FPN1, Ferroportin 1; [6]TFR1, Transferrin Receptor 1; [7]Ferritin, Ferritin

Iron deposition by Perls' Prussian Blue Staining

Our group has previously described that the human breast malignancy is characterized by a deregulation on the expression of iron-related proteins not only in epithelial cells, but also in lymphocytes and macrophages (Marques et al., 2014b). Given the fact that we have not found major differences in the expression of these proteins between benign and malignant mammary lesions of the cat and dog, we assessed iron deposition in the epithelial and stromal inflammatory cell compartments, to verify if this

could account for the absence of differences in the expression of proteins that regulate cellular iron homeostasis. In fact no differences were found, either for the epithelial or stromal inflammatory cells compartment, for the percentage of cases presenting iron deposition between benign and malignant tissues from cats and dogs ($p > 0.05$) (Fig. 2).

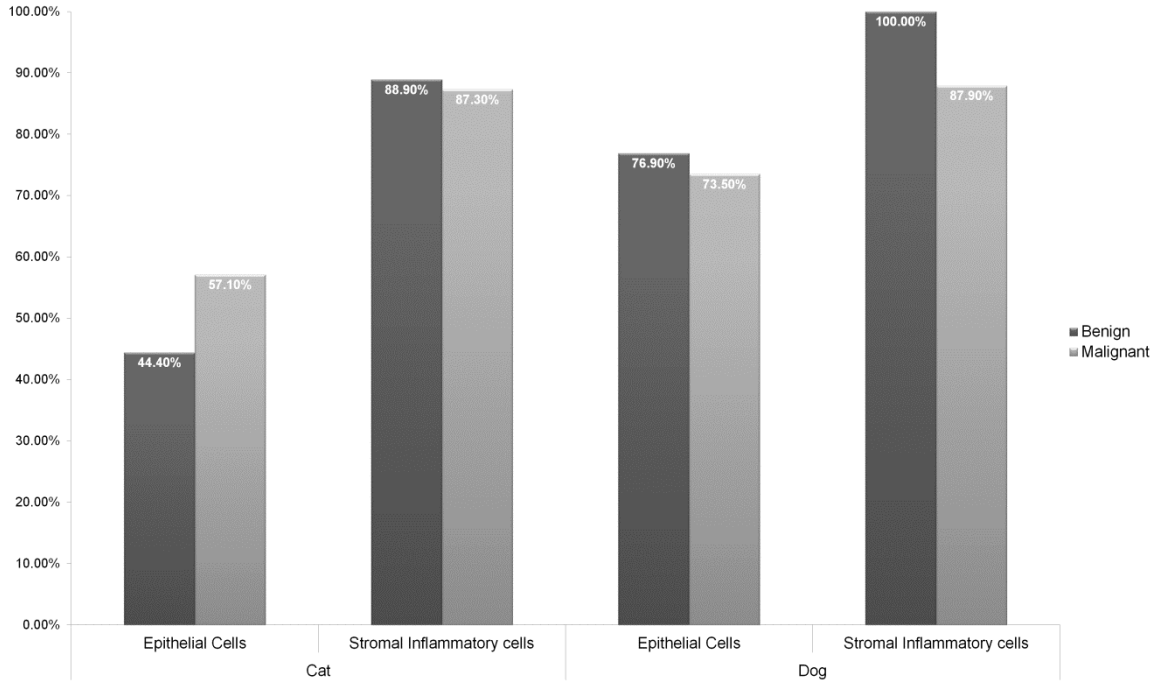

Figure 2. Iron accumulation in the mammary tissue of cats and dogs. The percentage of cases presenting hemosiderin deposition, as assessed by Perls' Prussian Blue staining, in epithelial and stromal inflammatory cells from benign and malignant mammary lesions from cats and dogs is shown.

Notably, Perls' Prussian Blue histochemical staining of the mammary gland samples from one necropsied dog and cat, without evidence of neoplastic disease, also revealed hemosiderin accumulation in epithelial cells and macrophages (Fig. 3A). Subsequent ultrastructural analysis allowed the identification of highly electron-dense Prussian Blue deposits within dense bodies, visible in both stained and unstained ultrathin sections (Fig. 3B,C). These iron-containing dense bodies, known as siderosomes, had an average diameter of about 0.5 µm and were located close to the nucleus of epithelial cells. In control samples without Perls' reaction, the highly electron-dense deposits were absent (Fig. 3D).

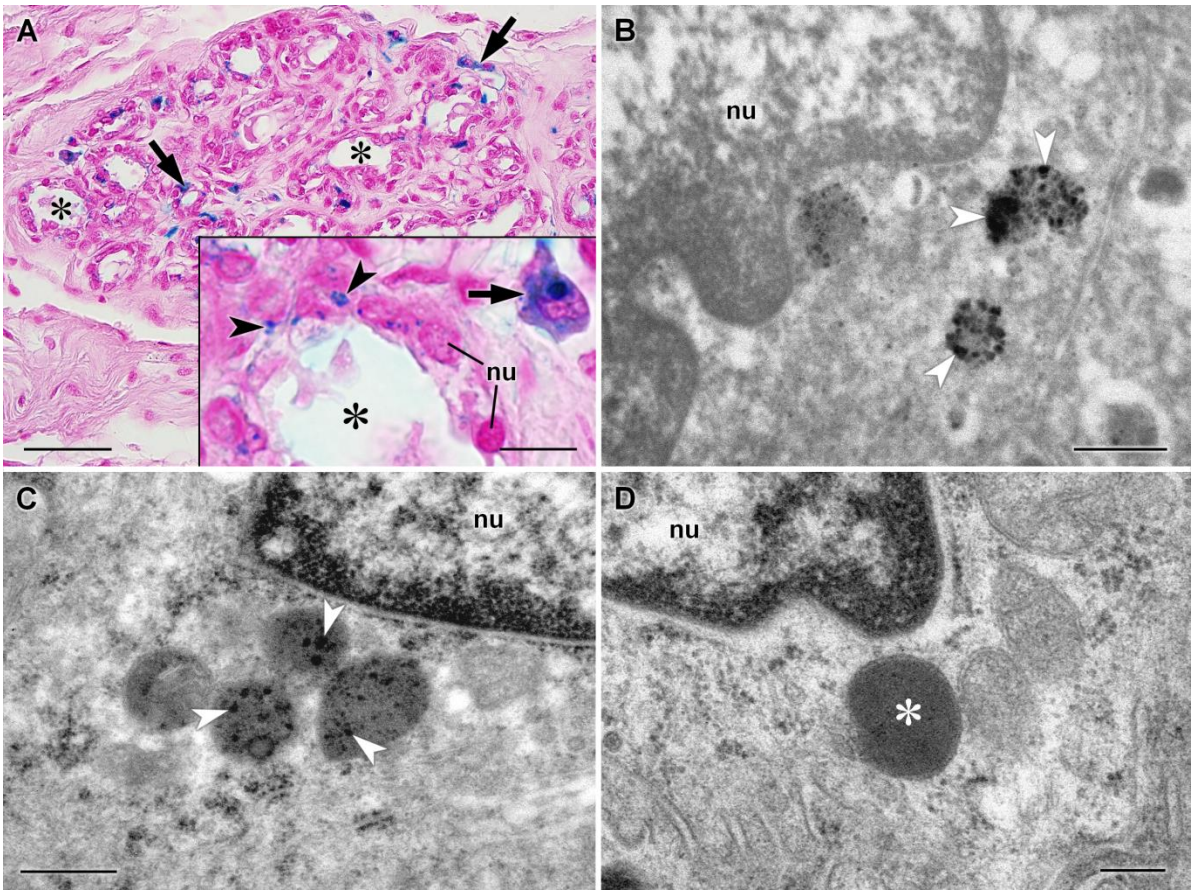

Figure 3. Light (A) and electron microscopy (B-C) detection of iron by Perls' Prussian Blue staining in a normal canine mammary gland. (A) Positive reaction for hemosiderin in macrophages (arrows) and in small granules (arrowheads) located close to the nucleus (nu) in epithelial cells of mammary ducts (asterisks) (Scale bar - 50 µm; Scale bar in inset - 10 µm); (B) Unstained ultrathin section showing highly electron-dense Prussian blue deposits (arrowheads) within siderosomes close to the nucleus (nu) in an epithelial cell (Scale bar - 0.5 µm); (C) Stained ultrathin section also revealing iron deposits (arrowheads) within siderosome (Scale bar - 0.5 µm); (D) Siderosome (asterisk) in a stained ultrathin section of a control sample without Perls' reaction (Scale bar - 0.2 µm)

Discussion

As a critical component of several sensor, transporter and storing molecules and enzymes responsible for energy production and intermediate metabolism (Ganz and Nemeth, 2011), iron is an essential transition metal for cell survival, growth and differentiation (Andrews, 2008). However, this constant requirement for iron becomes challenging in a way that the organism must acquire enough iron for its needs, while avoiding free iron toxicity (Andrews and Schmidt, 2007) and consequent increase in

oxidative stress and cell damage (Toyokuni, 1996; Mccord, 2004). Besides 'iron-overload' classical diseases such as hereditary hemochromatosis and thalassemias a growing body of evidence links imbalances of iron homeostasis with several chronic and neurodegenerative diseases (Kell, 2009).

Current knowledge supports the hypothesis that iron may affect the development, behavior and progression of cancer (Kwok and Richardson, 2002; Huang, 2008). Particularly in human breast cancer, several studies highlight that the malignant state is characterized by a deregulation of iron homeostasis, as shown by an altered expression of its regulatory proteins (Marques et al., 2014a). Human breast cancer epithelial cells are characterized by an 'iron-deficient' phenotype as demonstrated by the massive upregulation of the iron importer TFR1 (Faulk et al., 1980; Yang et al., 2001; Habashy et al., 2010; Singh et al., 2011) and lower levels of the iron storage protein ferritin (Rossiello et al., 1984; Alkhateeb et al., 2013) and ferroportin 1, the iron exporter, (Pinnix et al., 2010; Zhang et al., 2014) than with their normal counterparts. Overall, this appears to contribute to an increase of the metabolically available iron (labile iron pool), and proliferative and malignancy potential (Pinnix et al. , 2010; Zhang et al. , 2014). In this respect, besides the significantly lower ferritin expression in cats' malignant tumors, we were not able to replicate the results found for human breast cancer epithelial cells in cats' and dogs' mammary tumors. Additionally, our group has recently demonstrated that human breast tumor infiltrating lymphocytes and macrophages present an 'iron-donor' phenotype, characterized by a higher expression of both FPN1 and FT and a concomitant activation profile, as reflected by the also higher expression of hepcidin and TFR1 (Marques et al. , 2014b). Assessment of these iron-related proteins in tumor-infiltrating lymphocytes and macrophages from cats and dogs' mammary gland tissue also did not reflect human findings, suggesting that these cells do not present a particular 'iron-donor' phenotype in malignant samples.

Given the recognition that tumor cells are highly iron-dependent due to its proliferative activity (Sutherland et al., 1981; Trowbridge and Domingo, 1981; Taetle and Honeysett, 1987; Trinder et al., 1996;) at least a higher expression of TFR1 in tumor epithelial cells would be expected. As this was not observed, a plausible explanation could be that these cells were not 'iron-deficient'. With this in mind, we evaluated hemosiderin deposition in epithelial and stromal inflammatory cells in the same samples. The finding that 44% of all cat and 73% of all dog samples presented iron deposition in mammary gland tissue epithelial cells was surprising, contrasting with the much lower percentage that we previously found in human breast cancer specimens (~14%). But in contrast to the

human cohort, where a significantly higher percentage of breast cancer cases presented iron accumulation in epithelial and stromal inflammatory cells, when compared with reduction aesthetic surgery samples (Marques et al. , 2014b), in the present models, we found no evidence to support any relation between iron-laden macrophages and the dog and cat neoplasms. This suggests that cats and dogs' tumors do not need to alter the expression of iron-related proteins as an approach to acquire or maintain more iron for proliferating tumor cells, given that epithelial cells from normal or benign tissues already accumulate a sufficiently large amount of iron in comparison to what is seen in human breast tissues.

Breast epithelial cells that line the smaller ducts and alveoli of the mammary gland are responsible for the elaboration and secretion of a large part of milk components (Seelig and Beer, 1978; Ballard and Morrow, 2013). Cats and dogs secrete milk in which the concentration of iron is several times higher than in human milk (Keen et al., 1982). This suggests a higher retention of iron in cats and dogs' mammary tissue and hence providing a plausible explanation for the lack of additional iron requirements for tumor cells. Analysis of normal mammary gland tissue stained with Perls' Prussian Blue reinforced our light microscopy results by revealing the presence of iron-containing bodies, known as siderosomes, in the cytoplasm. Although we cannot clearly identify the origin of these siderosomes, we hypothesize that these are of lysosomal nature given that other authors have previously demonstrated that hemosiderin accumulates in lysosomes in macrophages and epithelial cells, such as hepatocytes and kidney epithelial cells (Dullmann et al., 1991; Jonas et al., 2002; Meguro et al., 2007).

The results discussed here do not imply that iron does not influence the development, behavior or progression of mammary gland carcinogenesis in the cat and in the dog. Actually, the fact that cats and dogs' mammary epithelial cells accumulate much higher iron concentrations than in humans' may also account for the high prevalence of cancer in those animals (Munson and Moresco, 2007), due to chronic cellular iron toxicity and oxidative stress (Nelson, 1992; Toyokuni, 1996; Eaton and Qian, 2002). In fact, estrogen and progesterone hormones are the single most important risk factor for the high incidence of mammary tumors in cats and dogs (Morrison, 1998; Baba and Câtoi, 2007). Previous studies have shown that estrogen metabolites in incubation with rat liver cytochrome P450 reductase and NADPH form superoxide radicals and reduce ferritin-bound Fe^{3+} to Fe^{2+}, potentially leading to the release of free, unbound iron (Wyllie and Liehr, 1997). This association between the redox cycling of catecholestrogen metabolites and the release of iron, through the generation of oxygen radicals (Liehr and Jones,

2001), has been proposed as a critical modulator of human breast cancer behavior and aggressiveness (Huang, 2008). It may also constitute an alternative explanation for the high hemosiderin accumulation in cats and dogs' mammary gland epithelial cells.

Our results are limited not only by the low number of cases, particularly of benign lesions in the cat mammary cohort, but ultimately by the fact that these were not representative of 'normal' mammary tissue, but of benign disease. One may argue that although histologically 'normal', benign alterations may already present several modifications that could mask real differences for the expression of cancer-intervenient proteins between samples. However, the electron microscopy studies were done in tissues from healthy animals, without any history of disease, and iron accumulation was clearly evident. Physiological differences in mammary iron uptake between species with high iron tissue levels, like the cat and the dog, and species with low iron levels, like the human species, should be clarified to verify if these animals are indeed suitable models of human breast cancer.

Conflicts of Interest

The authors declare they have no competing interests.

Author Contributions

Conceived and designed the experiments: OM, AC, EO, IA, FS, AG, ALC, BMS, GP, CL. Performed the experiments: OM, FF, EO, IA, ALC. Analyzed the data: OM, AC, FS, AG, ALC, GP, CL. Contributed with reagents, materials and analysis tools: FF, EO, FS, AG, ALC, BMS; GP, CL. Wrote the paper: OM. All authors revised and approved the final manuscript.

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Supplementary Table 1. Immunoexpression of iron-related proteins in feline mammary gland tissue, per type of lesion.

		Hepcidin (Mean ± SEM[1])	FPN1[2] (Mean ± SEM)	TFR1[3] (Mean ± SEM)	FT[4] (Mean ± SEM)
Hyperplasias	EC[5]	n= 5	n= 5	n= 5	n= 5
		3.80 (± 0.92)	10 (± 0.00)	0.90 (± 0.68)	11.57 (± 0.67)
	Ly[6]	n= 3	n= 2	n= 5	n= 2
		4.22 (± 2.12)	2.5 (± 0.50)	1.53 (0.76)	12.50 (± 0.00)
	M0[7]	n= 3	n= 2	n= 4	n= 4
		10.33 (± 1.67)	4.50 (± 0.50)	8.75 (± 1.25)	14.38 (± 0.63)
Fibroadenomas	EC	n= 3	n= 4	n= 4	n= 4
		1.33 (± 0.33)	7.88 (± 2.02)	2.25 (± 0.75)	11.25 (± 1.61)
	Ly			n=1	n= 1
				0.00	12.5
	M0			n=2	n= 4
				5.50 (± 4.50)	15.00 (± 0.00)
Tubulopappillary Carcinoma	EC	n= 31	n= 28	n= 31	n= 30
		1.66 (± 0.38)	9.76 (± 0.69)	1.56 (± 0.33)	9.47 (± 0.52)
	Ly	n= 16	n= 19	n= 26	n= 18
		2.19 (± 0.79)	3.45 (± 0.52)	0.57 (± 0.27)	9.68 (± 0.91)
	M0	n= 16	n= 19	n= 24	n= 25
		6.06 (± 0.80)	7.00 (± 0.73)	3.54 (± 0.74)	14.81 (± 0.13)
Solid Carcinoma	EC	n= 27	n= 23	n= 26	n= 26
		2.03 (± 0.60)	9.78 (± 0.63)	1.21 (± 0.24)	8.77 (± 0.50)
	Ly	n= 13	n= 14	n= 22	n= 16
		2.88 (± 0.81)	3.07 (± 0.47)	1.32 (± 0.38)	7.79 (± 0.78)
	M0	n= 16	n= 16	n= 22	n= 23
		7.69 (± 0.91)	7.93 (± 0.75)	3.00 (± 0.76)	15.00 (± 0.00)

[1]SEM, Standard Error of the Mean; [2]FPN1, Ferroportin 1; [3]TFR1, Transferrin Receptor 1; [4]FT, Ferritin; [5]EC, Epithelial Cells; [6]Ly, Lymphocytes; [7]M0, Macrophages

Supplementary Table 2 Immunoexpression of iron-related proteins in canine mammary gland tissue, per type of lesion.

		Hepcidin (Mean ± SEM[1])	FPN1[2] (Mean ± SEM)	TFR1[3] (Mean ± SEM)	FT[4] (Mean ± SEM)
Hyperplasias	EC[5]	n= 6	n= 5	n= 4	n= 5
		1.33 (± 0.85)	11.50 (± 2.21)	1.50 (± 0.50)	9.17 (± 1.44)
	Ly[6]	n= 2	n= 2	n= 4	n= 3
		1.00 (± 0.00)	6.67 (± 4.26)	0.38 (± 0.24)	4.67 (± 1.67)
	M0[7]	n= 1	n= 2	n= 4	n= 4
		8.00	9.00 (± 1.00)	6.75 (± 1.97)	13.75 (± 1.25)
Adenomas	EC	n= 11	n= 9	n= 11	n= 11
		1.80 (± 0.30)	9.23 (± 0.96)	3.27 (± 0.95)	10.45 (± 0.82)
	Ly	n= 4	n= 3	n= 7	n= 4
		2.38 (± 0.63)	4.77 (± 1.32)	1.04 (± 0.30)	11.50 (± 2.18)
	M0	n= 6	n= 4	n= 11	n= 9
		12.43 (± 0.95)	6.38 (± 1.52)	8.18 (± 0.76)	15.00 (± 0.00)
Benign MMT	EC	n= 8	n= 7	n= 9	n= 9
		1.81 (± 0.45)	10.12 (± 1.00)	3.00 (± 0.66)	10.00 (± 0.83)
	Ly	n= 4	n= 2	n= 8	n= 3
		1.00 (± 0.46)	7.00 (± 3.00)	0.67 (± 0.32)	8.67 (± 0.67)
	M0	n= 4	n= 3	n= 8	n= 9
		12.38 (± 1.68)	11.68 (± 1.67)	7.13 (± 1.12)	15.00 (± 0.00)
Tubulopappillary Carcinoma	EC	n= 12	n=11	n= 10	n= 10
		0.88 (± 0.35)	11.29 (± 1.13)	2.75 (± 0.42)	9.92 (± 0.69)
	Ly	n= 7	n= 7	n= 9	n= 6
		2.36 (± 0.98)	4.41 (± 1.16)	1.17 (± 0.40)	8.00 (± 0.89)
	M0	n= 5	n= 8	n= 9	n= 8
		6.80 (± 2.42)	6.88 (± 0.82)	7.78 (± 0.88)	15.00 (± 0.00)
Solid Carcinoma	EC	n= 11	n= 10	n= 11	n= 10
		2.31 (± 0.63)	11.15 (± 0.85)	2.32 (± 0.60)	9.75 (± 0.58)
	Ly	n= 5	n= 7	n= 9	n= 4
		2.70 (± 0.98)	5.06 (± 1.71)	1.17 (± 0.40)	10.25 (± 1.65)
	M0	n= 4	n= 8	n= 7	n= 11
		10.50 (± 1.94)	8.71 (± 0.84)	7.71 (± 0.87)	14.77 (± 0.22)
Complex Carcinoma	EC	n= 12	n= 10	n= 11	n=12
		1.17 (± 0.45)	9.33 (± 1.07)	2.15 (0.51)	10.56 (± 0.68)
	Ly	n= 8	n= 7	n= 10	n= 8
		3.35 (± 1.10)	4.94 (± 1.11)	1.12 (± 0.50)	9.39 (± 1.14)
	M0	n= 6	n= 8	n= 11	n= 12
		9.67 (± 1.87)	7.69 (± 0.88)	8.41 (± 0.70)	15.00 (± 0.00)

[1]SEM, Standard Error of the Mean; [2]FPN1, Ferroportin 1; [3]TFR1, Transferrin Receptor 1; [4]FT, Ferritin; [5]EC, Epithelial Cells; [6]Ly, Lymphocytes; [7]M0, Macrophages

Chapter 7

Discussion and Conclusions

7.1. General Discussion

Despite continuous development of novel diagnostic and technological tools for the management of breast cancer, many tumors still become chemo-resistant and progress to metastatic disease. This is when they become potentially fatal. Extensive data validates the fact that the accumulation of genetic and epigenetic mutations in breast epithelial cells is not enough to drive tumor progression, indicating a role for the host tissue microenvironment. The work presented throughout this thesis, by demonstrating that iron homeostasis deregulation is not restricted to breast cancer cells but also to stromal cells, adds new evidence to the hypothesized role of cells of the tissue microenvironment, such as lymphocytes and macrophages, as promoters of tumor progression through local regulation of iron homeostasis.

Several pathways have been proposed for iron-related carcinogenesis, such as cellular damage by iron-induced oxidative stress [1, 2] and iron-promoted cell proliferation [3, 4]. In the last few decades, breast cancer has seen a prolific growth in the knowledge of its iron metabolism. From the primordial studies describing serum and breast tissue ferritin content and the association with breast cancer risk and recurrence [5, 6], to the more recent ones unveiling the role of the ferroportin-hepcidin axis [7, 8], we have come a long way in unraveling the mechanisms behind tumoral epithelial cell iron regulation derangement. Overall, breast cancer epithelial cells present an ‘iron-deficient’ phenotype with an increased expression of proteins associated with iron acquisition [9-11] and lower expression of proteins linked to iron export and storage [7, 8, 12, 13], as it happens with other types of tumors [14, 15]. Here we describe an alternative ‘iron-utilization’ phenotype for breast cancer cells with simultaneous increased expression of TFR1 and hepcidin, and decreased expression of FT, which is consistent with a proliferative status in which iron availability is crucial. A recent shift in breast cancer has been occurring by focusing on the role of stromal cells. Given the fact that genetic and epigenetic alterations known to regulate breast morphogenesis are the main initiators of breast carcinogenesis and since most breast ductal cells are weakly invasive, both *in vitro* and *in vivo*, it is very likely that stromal cells might be, in part, responsible for facilitating the progression from pre-malignant lesions to invasive breast cancer [16, 17]. In this regard, the study of stromal inflammatory cells, as lymphocytes and macrophages, such important players in the regulation of systemic iron homeostasis, is lacking in the context of breast tumorigenesis [18]. Herein, we present, for the first time, a description of the iron-related phenotype of tumor-infiltrating lymphocytes and macrophages in breast carcinomas, and compare them with normal breast reduction tissue-resident counterparts. We demonstrated that, in

primary breast carcinomas, these cells present an 'iron-donor' phenotype as demonstrated by their higher expression of FPN1 and FT, while maintaining an activated profile seen by the increased expression of hepcidin and TFR1. These results are in concordance with the proposed idea that the surveillance role of the cells of the immune system against iron toxicity may favor tumor growth, either through supply of this critical element or by promoting angiogenesis [19, 20]. FPN1 was first discovered in 2000, by three independent groups [21-23], and shown soon after to be the receptor for hepcidin, and the only known iron exporter described to date [24, 25]. Since then, several groups have demonstrated that FPN1 may also be transcriptionally and postranscriptionally regulated by heme [26, 27], iron levels [26, 28-30], hypoxia [31-33] or even cytokines [34-36]. Our results, in primary breast tumors and metastized lymph nodes show a concomitant overexpression of hepcidin, FPN1, TFR1 and FT in lymphocytes and macrophages. Although unexpected, the simultaneous overexpression of these proteins is plausible mainly in neoplastic context and in a tissue with higher iron levels than normal counterparts that might modulate the phenotype of immune system cells. In a neoplastic setting where angiogenesis is promoted to supply nutrients and oxygen essential to tumor growth and metastasis, red blood cell incoming might trigger or maintain these alterations [37]. In fact, heme may be the responsible driver of this FPN overexpression, independently of hepcidin levels, at least in macrophages [25, 26]. Heme is also a potent transcriptional activator of FTL and FTH genes. Additionally, the secretion of ferritin by macrophages and lymphocytes, previously demonstrated by others [38, 39], may engage in a feedback loop that further promotes angiogenesis. Coffman and coworkers have shown that ferritin blocks the antiangiogenic effects of the cleaved high molecular weight kininogen (HKa) in endothelial cells, namely induction of apoptosis, inhibition of migration and inhibition of tube formation. Furthermore, they also demonstrated that ferritin restored migration and survival of HKa-treated cells *in vitro* and preserved tumor-dependent vessel growth in the presence of HKa *in vivo* [40]. These evidences advocate another role for ferritin, besides an iron storage or mitogenic protein, as a regulator of tumor-associated angiogenesis. Phenotypic characterization of breast tissue macrophages revealed an increased expression of CD163, a high-affinity scavenge receptor for hemoglobin-haptoglobin complexes [41], in carcinomas. The tumor-associated macrophages phenotype observed is consistent with the described for M2 macrophages by Recalcati *et al*. with an upregulation of FPN1 and resulting capacity to sustain malignant cell growth through iron release [20]. Although the phenotype is not fully recapitulated due to divergences in the expression of TFR1 and FT we cannot rule out the influence of tissue elements, as mentioned above, that are not reproducible *in vitro.* Regarding lymphocytes, although previous studies suggest a different ability to handle iron between CD4+ and

CD8+ T-lymphocytes [42, 43], we did not observe a particular lymphocyte profile associated with increased FPN1 and FT expression.

Considering that lymphocytes and macrophages may constitute 'iron-donor' cells in charge of tissue 'iron-nutrition', we asked if their 'iron-profiles' could also be deviated in case of metastasis. Overall, as for the primary tumor, we observed an increase in the expression of proteins related to iron export (FPN1 and FT) in lymphocyte/ macrophage areas of metastized lymph-nodes of patients with breast cancer, when compared with non-metastized ones. Lymph nodes are the most frequent sites for metastasis and there are probably several reasons for this, besides organ proximity, such as the lack of a basement membrane in lymphatic vessels and low pressure lymphatic fluid [44]. However, it is probable that cell-cell communication and secretion of factors may to some extent mimic what happens in the primary tumor, at least to allow the settling of the first metastatic cell. It is long known that not only malignant cells are capable of changing their microenvironment, but also that an abnormal stroma is capable of enhancing the malignant potential of epithelial cells [45]. So, it is a question of whether the metastatic cells hijack the control of stromal cells' iron metabolism in their favor by importing the iron released via-FPN1 or FT by them, or if a previously activated stroma (as seen in non-metastized lymph from patients with metastized breast cancer, with higher FPN1 expression than lymph nodes from patients with non-metastized breast cancer; unpublished results) may facilitate epithelial cell settling in a more favorable microenvironment [46, 47]. Therefore, the development of a pre-metastatic niche is, as a matter of fact, very similar to that of the primary tumor stroma, in which the progress of an inflammatory microenvironment co-evolves with the genetic and epigenetic changes in cancer cells [47, 48].

The fact that we found FPN1 overexpression in lymphocytes and macrophages to be as high in benign lesions of the breast as in ductal carcinomas (unpublished observation) led us to think that this mechanism may constitute a common physiological nutrition task (as 'iron-deliverers', once FPN1 expression is also observed, to a minor extent in reduction mastectomy specimens) or rather a tissue alteration more primordial than would be initially thought. In fact, there are several similarities in stromal-breast epithelial relationships between mammary gland development and breast carcinogenesis [49, 50]. Changes in the breast microenvironment can happen earlier than in DCIS stages where proliferating breast epithelial cells can secrete chemokines which may lead to the accumulation of leukocytes, mesenchymal stem cells, endothelial cells and fibroblasts [17, 51, 52]. This may often lead to inflammation and fibrosis (which are also involved in wound healing-response and exist in benign lesions before carcinoma) and induce even

more alterations in the breast microenvironment, ultimately driving breast tumorigenesis [53, 54]. In this sense, in precursor lesions, a more reactive stroma in which stromal inflammatory cells actively export more iron, can not only promote the proliferation (aberrant or not) of breast epithelial cells but also the development of alternative stromal-cell alterations related to the deposition of iron in the tissue.

The fact that precursor lesions already display iron-related alterations in the tumor microenvironment (either as a cause or consequence) is also patent in our second study of the association between CCL2 expression and the modulation of tissue iron levels in the breast. We demonstrate that epithelial and stromal inflammatory cells from non-neoplastic breast tissue microenvironments, adjacent to carcinomas, already present iron accumulation, unlike breast aesthetic reduction specimens. Iron accumulation on stromal inflammatory cells, in turn, is associated with the expression of CCL2 in breast epithelial cells. CCL2 is a chemotactic cytokine with a role in breast cancer progression through an established positive feedback loop mechanism with macrophages that promotes angiogenesis, epithelial cell migration and metastasis [55-58]. CCL2 expression had been previously described to be influenced by cellular iron levels and modulated by HFE variants [59-63]. Recently, our group has demonstrated that CCL2 is an inhibitor of TFR1 expression and that Ccl2-/- mice present elevated iron levels, splenic iron overload and mild iron accumulation in the liver, due to hepcidin-independent reduction of ferroportin levels [64] (unpublished results). Thus, epithelial cell secretion of CCL2, even in precursor lesions, may recruit leukocytes to the tissue milieu that can further support epithelial cell proliferation, either by recruiting more leukocytes, as reflected by the association between CCL2-positive macrophages and total macrophage infiltration, and/ or by iron delivery. Overall, these results add evidence to the proposed paracrine signaling interplay between epithelial cells and macrophages, where overexpression of CCL2 may be responsible for the attraction of iron-loaded leukocytes and consequent iron export to the tissue microenvironment.

In addition, our results highlight another layer of iron-related regulation by backing up studies that suggest that HFE variants may modulate neoplastic cell behavior [65, 66]. Since HFE variants are highly prevalent, the possibility of understanding how they influence breast cancer progression, risk and treatment is of extreme importance. The hypothesis that HFE variants are associated with breast cancer aggressiveness lies on the observation that the lack of a functional HFE protein product increases iron absorption and favors tumor oxidative stress and genomic instability [67, 68]. In our study we provide a possible explanation for HFE variant regulation of breast cancer behavior by showing that p.C282Y heterozygous IDC patients present a significantly higher expression of

hepcidin in lymphocytes and macrophages than wild-type and H63D carrier patients and that p.C282Y/p.H63D compound heterozygotes also display a significantly higher expression of TFR1 in all the cell types analyzed, which may promote increased intracellular iron content. However, in our study no associations were found between HFE variants and clinicopathological markers of breast cancer progression and prognosis, therefore precluding any important role of HFE variants as biomarkers in breast cancer management. Nevertheless, despite much of the focus on HFE and cancer being linked to its regulation of iron homeostasis, a connection with the immune system cannot be disregarded. For instance, hereditary hemochromatosis patients present significantly lower lymphocyte counts [69] and disease severity is correlated with CD8 T-lymphocyte numbers [70, 71]. Furthermore, recent studies suggest that HFE acts as a negative regulator of CD8 T-lymphocyte activation [72] and influences CD8 T-cell differentiation and maturation [73]. On the other hand, activated T-lymphocytes are able to downregulate HFE expression in tumor cell lines, through production of TNF and IFN-γ [74]. In this sense, our results on the overexpression of hepcidin and TFR1 on lymphocytes and macrophages add relevant information for the view on how HFE may influence the cells of the immune system in a specific neoplastic context.

Animals develop many diseases that also affect humans, including cancer. For this reason, animal models in which spontaneous malignant tumors arise constitute an important and valuable tool for cancer studies. In the end, the main objective is to understand key factors for the disease initiation and progression that may eventually help in the development of new therapies. Advantages for the use of cats and dogs in breast cancer studies over other animals include: (1) the fact that they are outbred animals, contrarily to some inbred mice and rats; (2) mammary gland tumors are spontaneous and not induced; (3) tumors are histologically similar and respond comparably to conventional therapies; (4) they share environmental factors with humans; (5) malignant tumors develop more rapidly due to their shorter life expectancy; (6) clinical trials produce more results due to a shorter life expectancy [75]. Furthermore, several underlying genetic and cytogenetic alterations were demonstrated to be common denominators between feline and canine mammary gland tumors and human breast cancer [76]. Nonetheless some doubts remain as to whether results obtained in pets' mammary gland tumors are transferable to human breast cancer, due to differences such as the high proportion of feline mammary gland tumor's hormone receptor negative when compared with its human breast counterparts [77, 78]. Regarding this, we dissected the iron-phenotype of breast epithelial cells, lymphocytes and macrophages from cats and dogs' benign lesions and mammary gland tumors. Unexpectedly, we found no significant differences in the

expression of iron-related proteins between lesions, that could be explained by the accumulation of iron in normal mammary gland and benign lesions. Given this fact, our results add evidence to the fact that cats and dogs may not be good comparative oncology models for human breast cancer, at least regarding the deregulation of iron homeostasis.

Overall, the results described in this thesis demonstrate that the deregulation of iron homeostasis in human breast cancer is a common denominator in several cell types, and that the expression of iron-related proteins in these cells is associated with clinicopathological markers of breast cancer behavior and progression. In this sense, it becomes increasingly critical to acknowledge the role of stromal cells in breast carcinogenesis and deepen the knowledge concerning the interactions with epithelial cells in order to clarify what these new prognostic markers indicate in biological context.

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7.2. Conclusions and Future Perspectives

In this thesis we report, for the first time, the expression of iron-related proteins in lymphocytes and macrophages from the breast tissue microenvironment. We demonstrate that breast ductal carcinoma infiltrating lymphocytes and macrophages present an 'iron-donor' phenotype, as observed by its higher FPN1 and FT expression than in aesthetic reduction specimens. Strikingly, this phenotype is also observed in metastized lymph-nodes. Additionally, markers of breast cancer behavior and prognosis were shown to be associated with the expression of iron-related proteins not only in epithelial cells but also in lymphocytes and macrophages. Once FPN1 expression in these stromal inflammatory cells is not associated with hepcidin expression nor iron accumulation, we hypothesized that CCL2, a chemokine previously shown to modulate iron regulation (and vice-versa), could be modulating tissue iron levels in the breast. In fact, an increase in the expression of CCL2 in epithelial cells was correlated with higher FPN1 expression in lymphocytes. Although we did not observe any modulation of CCL2 expression in epithelial cells or macrophages by HFE polymorphisms, as previously reported, we did recognize an increased expression of hepcidin and TFR1 (in all the cell types analyzed) in HFE p.C282Y/p.H63D compound heterozygous patients, which can provide a possible explanation for the previously reported findings of a more aggressive course of disease in p.C282Y carriers. Finally, we concluded that cats and dogs do not seem to be good animal models for the study of iron homeostasis in human breast cancer since they do not recapitulate the differences in the expression of iron-related proteins between normal/benign and carcinoma tissue. Nevertheless, this study gave us the opportunity to describe, for the first time, the remarkable iron accumulation in normal epithelial cells from cats and dogs' mammary gland, unlike the normal human breast.

We believe our results are of relevance, not only in terms of advancing knowledge about iron metabolism regulation in a particular tissue context, but also the acknowledgment of previously unforeseen roles of the tissue microenvironment inflammatory cells in the tumor context. In spite of the advances, several questions remain to be answered and points to be proven.

It is of mounting importance to continue exploring the consequences of the cellular 'iron-donor' phenotype, by performing functional essays in co-culture or three dimensional cell systems. Furthermore, extending our results by verifying the impact of CCL2 neutralization on mammary gland iron levels and FPN1 expression in lymphocytes would add support to our view on the paracrine signaling interplay between malignant epithelial

cells and macrophages, where CCL2 overexpression is responsible for the recruitment of iron-loaded leukocytes that may increase tissue iron-loading through overexpression of FPN1.One must not forget, also, the pleiotropic roles of ferritin. The fact that ferritin expression is increased not only in carcinoma-infiltrating lymphocytes but also in metastized lymph-nodes, and is not associated with iron accumulation, suggests an alternative role for ferritin apart from its iron storage properties.

The breast cancer patient cohort should be extended in order to validate the results observed in p.C282Y heterozygous breast cancer patients. Given that p.C282Y/p.H63D compound heterozygous patients present a higher expression of hepcidin and TFR1, and this phenotype is associated with a more aggressive disease behavior, it should be worthwhile analyzing if HFE variants have a real impact in breast cancer progression and prognosis in larger multicentric studies.

Of particular interest is the fact that current cancer-related therapies include two, if not antagonizing, at least, competing views: (1) on one side iron-chelation is increasingly studied as a possible therapeutic alternative. In fact, given our results, iron chelation would be expected to impair malignant cell proliferation not only by direct limitation of iron bioavailability but also by blocking iron-delivery by cells of the tissue microenvironment; (2) on the other, iron replacement therapy is recommended in patients with cancer-related anemia. Thus, if circulating immune cells are capable of taking up iron and deposit it in other tissues, to what extent are we assisting the tumor, an entity capable of recruiting leukocytes in its own favor? In this sense, a deeper knowledge on the consequences of intravenous iron administration and how tissue microenvironment cells contribute to local iron regulation is of vital importance.

Finally, the recognition that normal epithelial cells from cats and dogs' mammary gland accumulate iron, unlike in the normal human breast, demands a deeper insight into the functional consequences and influences upon long time exposure.